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MAGELLANIC CLOUDS, VI. REVISED DISTANCES AND LUMINOSITIES

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Communicated March 2, 1953

New measures of the magnitudes and colors of globular clusters in the Magellanic Clouds have led to a rediscussion of the distances of these two galaxies. The revision suggested below has a bearing on all extra-galactic measuring, and therefore some general considerations are presented before the cluster data are discussed.

1. Ever since Hubble's identification of certain objects in the Andromeda Nebula as probable globular clusters[1] gave support to our tentative identification of several of the clusters in the Magellanic Clouds as globular,[2] we have been uncomfortable about the discrepancy, discussed by Hubble and others, between the computed average absolute magnitude of these other-system globular clusters and the magnitudes of those in our Milky Way system. Why should our globular clusters be about a magnitude brighter? There were three possible explanations.

(*a*) We first suspected the photometry of these difficult objects in all three systems. Some years ago I carried through a revision of the distances of the thirty high-latitude globular clusters of the Milky Way[3] to see if the discrepancy would be alleviated, but it was not. With the improved apparent magnitudes by Christie,[4] who used a *schraffierkassette* method, and with a general revision of the photometry of each cluster (the work of many individuals), we came out with much the same values as originally determined. The RR Lyrae variables in these high-latitude clusters give acceptable values of the distances, and the intervening space absorption is in all cases small and easily handled through the study of the frequency of background galaxies. Also various checks and revisions of the apparent magnitudes of the clusters in the Magellanic Clouds[5] suggested nothing seriously wrong; and similarly for the Andromeda Nebula, the revision of the magnitude scale and adjusted corrections for space absorption have not removed the large discrepancy.[6]

(*b*) A second explanation, at first too easily adopted, was simply that the globular clusters in irregular galaxies like the Magellanic Clouds need not be closely comparable with the globular clusters of our galaxy, and there is no uniformity requirement. But the Andromeda Nebula is apparently just like our system in type, much the same in mass, and therefore the large systematic difference for the clusters of the two spirals is difficult to accept.

(*c*) A third possible solution is of course to take the absolute magnitudes of globular clusters as actually comparable in all three systems and to decide, therefore, that the classical cepheids, on which the distances of the Clouds and the Andromeda Nebula had been based, are brighter than we had computed. That is, we assume that the zero point for the period-luminosity relation for the classical cepheids, in the Magellanic Clouds and elsewhere, is wrong. If, for example, we should increase by 1.3 magnitudes the absolute luminosities used in the period-luminosity relation of classical cepheids, thus nearly doubling the distances of the Clouds and the Andromeda Nebula, the absolute magnitudes of their globular clusters would be in essential agreement with the values for the local globular clusters.

2. This third accounting for the discrepancy is the one now accepted as justified by the best evidence available. We know roughly how much the alteration should be, but it may require several years of investigation of magnitude systems, globular cluster luminosities, variables in all branches of the cepheid family, and the motions and distances of the galactic cepheids, before an accurate adjustment factor can be established. In this communication we present the evidence provided by the globular clusters of the Magellanic Clouds.

Not only the magnitude discrepancies for globular clusters, but several other factors have contributed to the long doubt which is now leading to the large revision. For example, Baade's failure to isolate easily with the Hale Reflector the individual stars in the globular clusters of the Andromeda Nebula is significant. If the old distances were approximately correct, we should be able to photograph many individual stars at magnitude 21. If the distances were doubled, the giant stars of the globular clusters would appear only at magnitude 22, or fainter. Another example is the failure of the persistent Harvard searches for cluster-type cepheids in the Magellanic Clouds and in their globular clusters at magnitudes 17 to 18, where they should appear if the distances are the heretofore accepted eighty thousand light years. If on the other hand the distances are a hundred and fifty thousand light years, we must look for those short-period cepheids in the Clouds at magnitude 19.

3. For some time there has been a growing skepticism about the luminosities of the classical cepheids because the zero point of the period-luminosity curve, based on the proper motions of classical galactic cepheids,

seemed insecure. Dr. Baade presented several of the arguments for revision at the Rome meeting of the International Astronomical Union, September, 1952. The measured proper motions of these supergiant stars are not large compared with the motions of the comparison stars; perhaps they are not strongly dependable. The early work of Hertzsprung and Shapley on the motions of a few of the bright cepheids gave us our first zero point for the period-luminosity curve; and the much more extensive study by R. E. Wilson seemed to confirm it. Many others have worked on the proper-motion data with varied results, and in general the absolute magnitudes originally derived have been accepted, with reservations, and

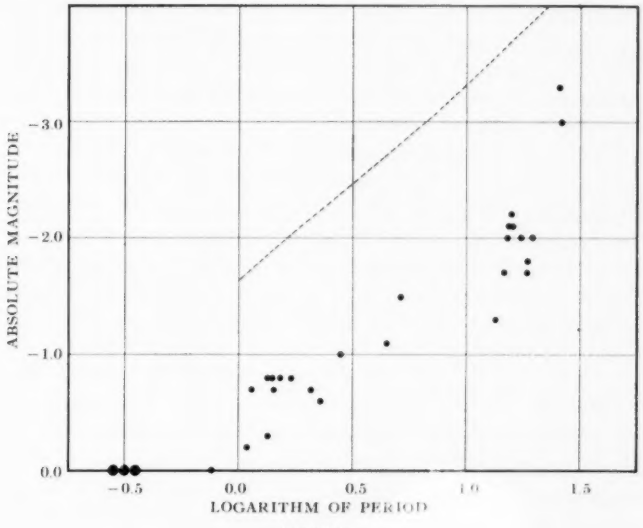

FIGURE 1

Period-luminosity relation for cepheids in globular clusters

almost universally used. The RR Lyrae cepheids, with relatively large motions, yield a sounder zero point, and for globular clusters, and the individual RR Lyrae variables, they give us distances reasonably free of doubt.

In the past year or so Baade, Mrs. Gaposchkin, Hubble, Kron, and others either have questioned the derived absolute magnitudes of the classical cepheids, or have contributed important measures, or made suggestions that lead toward the revision of the extragalactic distances and dimensions. For example, Joy[7] and Sanford[8] have given special attention to the W Virginis type of cepheid, which appears in globular clusters but

apparently not in the Clouds. In spectra, light curves, as well as in distribution, these somewhat abnormal variables differ from classical cepheids. But in the globular clusters they too show a dependence of luminosity on period. Our faith in the old zero point for classical cepheids was considerably fortified by the observed fact that several globular clusters contain long-period cepheids, which, together with their numerous cluster-

TABLE 1

THE BRIGHTER VARIABLE STARS IN THIRTEEN CLUSTERS

CLUSTER	VARIABLE	ABSOLUTE MAGNITUDE	LOG PERIOD
M 2 (7089)	1	−2.1	1.19
	5	−2.0	1.24
	6	−2.0	1.29
M 3 (5272)	95	−1.4	2.01
	154	−2.0	1.18
M 4 (6121)	29	−0.2	0.04
M 5 (5904)	42	−3.3	1.41
	50	−1.4	2.02
	84	−3.0	1.42
M 10 (6254)	2	−1.8	1.27
M 12 (6218)	1	−2.1	1.19
M 13 (6205)	1	−0.8	0.16
	2	−1.5	0.71
	6	−0.7	0.32
	8	0.0	−0.12
M 14 (6402)	1	−1.7	1.27
	2	−1.0	0.45
	7	−1.3	1.13
M 15 (7078)	1	−0.7	0.16
M 22 (6656)	11	−0.8	0.23
M 56 (6779)	1	−0.8	0.18
	3	−1.6	2.01
M 80 (6093)	1	−2.2	1.20
ω Cen (5139)	29	−1.7	1.17
	42	−1.0	2.17
	43	−0.7	0.06
	48	−1.1	0.65
	60	−0.8	0.13
	61	−0.6	0.36
	92	−0.3	0.13

type variables, show a definite period-luminosity relation. It was natural to equate this relation, obtained directly from half a dozen globular clusters, with the period-luminosity relation so extensively studied by us in the Magellanic Clouds and also worked up by Hubble for the Andromeda Nebula, Messier 33, and NGC 6822, and by Baade for IC 1613.

4. Figure 1 shows a plot of the periods and absolute magnitudes of the 25 cepheids, with periods longer than a day, found in 13 globular clusters

(table 1); one with a period of $0^d.75$ in Messier 13 is also included. The material is taken from Mrs. Hogg's catalog,[9] supplemented by data from Joy's important study of the spectra.[10] The suspected and proved RV Tauri stars are not included. Four variables with periods greater than 100 days are not plotted. They may be typical long-period variables of high luminosity.

The distances and the total absolute magnitudes in the clusters that contribute to figure 1 have been derived from the apparent magnitudes of their RR Lyrae cepheids, which are symbolically plotted at $M = 0$. Our present view is that the long-period cepheids in clusters, perhaps with two or three exceptions, are definitely of the W Virginis type; they are what we might call discoid cepheids (type II population), as distinguished from the classical "cloud" cepheids (type I), which are found in the Magellanic Clouds and are associated with the spiral arms of our own and other galaxies. The individual points scatter rather widely. These deviations may be lessened by the more precise work that is under way at Mount Wilson-Palomar.[11] Alternatively, the scatter may be inherent in this type of cepheid, which is also otherwise unusual.

Accepting the revision derived below for the absolute magnitudes of classical cepheids, we must draw the period-luminosity curve applicable to them roughly parallel to that for the variables in the globular clusters, and approximately 1.3 magnitudes above. The dotted line in figure 1 suggests the new position on the absolute magnitude scale. We naturally ask if there are intermediates in luminosity and spectral behavior, and if the Magellanic Clouds, for instance, have both types of the longer period cepheid variables.

5. The Small Cloud contains several "sixteen-day" cepheids. In our own galaxy such periods are common among the W Virginis type variables. Does this situation suggest that the Small Cloud (like our own galaxy) has both discoid and cloud cepheids in its population? The sixteen-day cepheids in the Small Cloud, however, fit on the period-luminosity curve that suits the classical cepheids. And a comparison of our work on the light curves in the Clouds[12] with S. Gaposchkin's light curves of W Virginis,[13] and with other light curves of variables of this type, shows a persistent difference. The rounded maxima and secondary "hump" are characteristic of the galactic system's W Virginis variables. The Small Cloud's sixteen-day cepheids, with scarcely any exceptions, have more normal curves.

The Large Magellanic Cloud has been considered, especially by Baade, as probably our purest example of population I; but if its globular clusters are like ours, we have again in that irregular galaxy a suggestion of a mixture of the presumably very young supergiant stars and of the old, perhaps primitive, population that is characteristic of globular clusters,

spheroidal galaxies, and the discoids and nuclei of spirals. The Clouds continue to present challenging questions.

One-third of all the long-period cepheids so far found in globular clusters fall in the relatively narrow interval from 13.5 to 19.5 days; the period of the type-star W Virginis is 17.27 days. A similar restriction in period is shown for the cepheid variables of the galactic nucleus,[14] but much less conspicuously if at all for the core of the Small Magellanic Cloud.[15]

6. Returning now to the globular clusters in the Magellanic Clouds, we note that a revision of the apparent magnitudes has become possible at this time for three reasons:

(*a*) We have received from the Boyden Station small-scale photographs suitable for estimating the total magnitudes of the clusters through direct comparison with stars of a stellar magnitude sequence. These plates are made with a Ross camera of 1.25 inches aperture and a focal length of 6.0 inches.

(*b*) A photoelectric magnitude sequence in the blue and yellow has been set up for the Large Magellanic Cloud by Dr. Uco van Wijk,[16] using the Rockefeller reflector at the Boyden Station. Dr. Ivan King has made a similar sequence (unpublished) for the Small Cloud. The photoelectric sequence differs very little (in the blue) from that established photographically for our variable star work through intercomparisons with northern standards. As a result, the new cluster magnitudes, based on estimates made on three plates by Mrs. Nail, differ little on the average from the values derived in past years by Harvard observers.

(*c*) Photoelectric colors for a number of the clusters have been determined by Gascoigne and Kron,[17] working with the 29-inch Reynolds reflector on Mount Stromlo. This work provides a check on the colors that we have inferred from the spectral classifications by Miss Cannon. Many years ago in the Henry Draper Extension,[18] Miss Cannon provided a fair discrimant between open and globular clusters for many objects in the Large Cloud.

7. A cluster is considered to be globular, and not merely a circular or symmetrical open cluster, if its integrated color index is $\geq 0^m.30$ or if its spectral class is F or later. There is, of course, a chance that occasionally a superposed yellow or red star might erroneously, through spectrum pollution, transfer a cluster from the open to the globular category; also that some true globular clusters are excluded because of interference in their composite spectra by superimposed early type stars. Examination of the texture of the cluster images on the reflector plates, however, can usually discriminate between the two types, thus supplementing the color criterion. (On the basis of structure as shown on blue, red, and infra-red plates, NGC 1856 is retained in the list (table 2), notwithstanding the bluish photoelectric color by Gascoigne and Kron, which has been recently

confirmed by photoelectric measures made at the Boyden Station by Mrs. Elske v. P. Smith; two rather bright superimposed stars appear to influence strongly the composite color.)

8. The median of the apparent magnitudes of the clusters in table 2 is $\dot{m} = 11.3$; the mean is $\bar{m} = 11.1 \pm 0.2$. The spread is considerable, but less than that observed among the thirty high latitude globular clusters of our galactic system. For the latter the median photographic absolute magnitude is $\dot{M} = -7.3$ and the weighted mean is $\bar{M} = -7.3 \pm 0.2$. The unweighted mean for the twenty best determined values is $\bar{M} = -7.36 \pm 0.2$ and the median, $\dot{M} = -7.45$.

If we equate the luminosities of the thirteen clusters of the Clouds (treated together) to the luminosities of our thirty (and also twenty) globular clusters, we obtain the distance modulus, $m - M = 18.6$, with

TABLE 2

CLUSTERS IN THE MAGELLANIC CLOUDS

NGC	APPARENT MAGNITUDE (VN)	APPARENT MAGNITUDE (G&K)	SPECTRUM (HARVARD)	COLOR (G&K)
121	11.4	11.44	G5	+0.70
416	11.7	11.86	K?	+0.50
419	10.1	10.62	G	+0.54
1783	10.8	10.55	F5:	+0.48
1806	11.6	11.15	G:	+0.60
1835	10.3	10.58	F8	+0.56
1846	11.2	10.86	G5	+0.58
1856?	9.9	9.96	G:	+0.20
1916	11.3	...	A7	...
1978	10.8	10.59	G	+0.64
2019	11.6	...	F5	...
2056	11.7	...	G	...
2121	12.0	11.57	..	+0.72

an estimated mean error of two- or three-tenths of a magnitude. The value based on the former zero point is 17.3.

Adopting a correction of $\Delta m = 0.3$ for space absorption, we obtain for the revised distance of the Clouds 150,000 light years. The new over-all diameters would be, with this revision, 30,000 l.y. and 20,000 l.y. for the Large and the Small Cloud, respectively. Since the clusters listed in table 2 are mostly from the Large Cloud, and since the adopted value of Δm seems appropriate for that Cloud,[19] which lies in a highly rich field of remote galaxies, the modulus and distance given above could best be associated with it. Additional studies will evaluate whatever may be the difference in distance for the Small Cloud.

9. The revision of the distance for the Magellanic Clouds leads to several important consequences. The luminosities of their supergiant stars are 1.3 magnitudes brighter, and the dimensions of their super-

voluminous red variable stars, recently studied at Harvard,[20] are of course increased. The P cygni variable S Doradus must now be estimated as more than a million times as bright as the sun. Scores of stars in both Clouds are more than one hundred thousand times the sun's luminosity. The brightest of the reddish cepheids, long-period stars, and irregular variables may have diameters comparable with that of Saturn's orbit.

An interesting consequence, if the revision is accepted, is that the classical cepheids of the galactic system, as well as those outside, are larger than heretofore computed, with the probable result of a decrease in the estimated mean density. This decrease should be to the advantage of the theory of cepheid variation,[21] where the relatively high value of the mean density of a typical cepheid has been something of an obstacle that may now be lessened, if not removed entirely. Another consequence is that the estimated distances of almost all external galaxies will be increased in the same proportion as the Magellanic Clouds. The Andromeda Nebula at a distance of 1,500,000 light years is nearly doubled in extent and may be even larger than our own system. Throughout metagalactic space there must be many galaxies as large and massive as ours, even though it remains definitely in the class of giants. The gap in linear dimensions between the greatest of globular clusters and the smallest of the dwarf galaxies that have so far been recognized is definitely widened,[22] since the revision of the zero point of the period-luminosity curve for classical cepheids does not affect measures of the distances and diameters of our globular clusters, or of other distances within the Milky Way system, except, of course, the distances of the classical cepheids themselves.

With the revision of extragalactic distances, the speed of expansion of the metagalaxy at a given distance is cut in two, approximately, and the "age" of the expansion is doubled. The time available for the stellar evolution in spheroidal galaxies, which might account for the puzzling Stebbins-Whitford effect, is much extended, thus giving more weight to that interesting suggested interpretation. And of course our own galaxy could have experienced more rotation, if the age of spiral galaxies is similarly extended, and could have had more time for dynamical adjustments than was permitted by the unrevised zero point.

Perhaps the most important contribution arising from the revision lies in the harmonizing of the geological and cosmic time scales. Evidence had increased of late, from radioactive measurements of the oldest rocks, that the earth's crust must be three or four thousand million years old, rather than half that value as suggested by the accepted expansion rate. Now we are in agreement again with the geologists and need not appeal to the hypothesis that the earth may be older than the expansion.

There will be other important consequences, especially in theoretical work, of a major revision of the zero point of the period-luminosity curve

for classical cepheids, but until the observational basis is further developed they need not be too seriously considered. It is sufficient now to note how many seeming discrepancies will be attenuated or removed by the revision, and how little argument there is against the change.

[1] Hubble, E., *Mt. Wilson Contr.* 452 (1932); see also Seyfert, C. K., and Nassau, J. J., *Ap. J.*, **102,** 377–381 (1945) for Baade's additions; and Mayall, N. U., and Eggen, O. J., *Pub. Ast. Soc. Pac.*, **65,** 24–29 (1953).

[2] Harvard Monograph No. 2 (1930). See also: *Harv. Obs. Bull.* 775 (1922); 868 (1929); 889 (1932); 895 (1934); *Harv. Obs. Circ.* 271 (1925).

[3] These PROCEEDINGS, **30,** 61–68 (1944): *Harvard Reprint* 257.

[4] Christie, W. H. *Mt. Wilson Contr.* 620 (1940).

[5] *Harv. Obs. Bull.* 889, 15 (1932).

[6] Baade, W., *Mt. Wilson Contr.* 696, 5 (1944); Seyfert, C. K., and Nassau, J. J., *Ap. J.*, **101,** 179–186 (1945).

[7] Joy, A. H., *Mt. Wilson Contr.* 578 (1937).

[8] Sanford, R. S., *Pub. Ast. Soc. Pac.*, **61,** 135 (1949).

[9] Sawyer, H., *Pub. David Dunlap Obs.*, **1,** no. 4 (1939). She called attention to the fragmentary period-luminosity curves in half a dozen of these clusters, *Ibid.*, no. 11, 238 (1942).

[10] Joy, A. H., *Ap. J.*, **110,** 105–116 (1949).

[11] Baade, W., *Pub. Mich. Obs.*, **10,** 7–17 (1951).

[12] These PROCEEDINGS, **26,** 541–548 (1940); *Harvard Reprint* 207.

[13] Gaposchkin, S., *Harv. Obs. Bull.* 906 (1937).

[14] These PROCEEDINGS, **26,** 681–688 (1940); *Harvard Reprint* 214.

[15] These PROCEEDINGS, **28,** 200–204 (1942); *Harvard Reprint* 241; *Ibid.*, **26,** 108, figure 4 (1940); *Harvard Reprint* 192; *Sky and Telescope*, **11,** 275 (1952).

[16] van Wijk, U., *Harv. Obs. Bull.* 921 (1952).

[17] Gascoigne, S. C. B., and Kron, G., *Pub. Ast. Soc. Pac.*, **64,** 196–200 (1952).

[18] Cannon, A. J., *Harv. Ann.*, **100,** 206 (1936).

[19] See discussion of color in following paper of this series, these PROCEEDINGS **39,** 358–362 (1953).

[20] *Ibid.*, **37,** 138–145 (1951). *Harvard Reprint* 346.

[21] Pointed out by L. Aller and M. Schwarzschild when this paper was presented at the Amherst meeting of the American Astronomical Society, December, 1952. Abstract, *Ast. J.*, **56,** 139–140 (1953).

[22] Baade, W., *Mt. Wilson Contr.* 697, 3 (1944).

MAGELLANIC CLOUDS, VII. STAR COLORS AND LUMINOSITIES IN FIVE CONSTELLATIONS

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Communicated March 2, 1953

The constellations of dominantly blue supergiant stars represented in our galactic system by the configurations of Orion, Scorpius, and Centaurus appear to have their counterparts in the Large Cloud of Magellan. The recent establishment of photoelectric magnitude sequences in the Magellanic Clouds has permitted an examination of the nature of several of the most important of these stellar aggregations.[1] The similarity to the Orion-type constellations is fully confirmed. Emission nebulosity and bright-line stellar spectra are in common. The stellar colors and constellation dimensions are alike. The excessive absolute luminosities, which are certain for the Large Cloud, may not often be attained in our own constellations.

1. Magnitude standards in the blue and yellow were set up photoelectrically for the stars of the Large Magellanic Cloud by Dr. Uco van Wijk,[2] working with the Rockefeller reflector at the Boyden Station of

TABLE 1

CONSTELLATION	NGC	AREA	BLUE RI ≤ -0.1	RED RI $\geq +3.0$
I	1935	$26' \times 26'$	14	2
II	1966	$17' \times 17'$	22	2
III	1974	$28' \times 28'$	32	2
IV	1869	$33' \times 33'$	15	2
V	1910	$24' \times 24'$	30	1

the Harvard Observatory. He established magnitudes for the brighter stars in Sequence V, which we have for many years used as the basis of our photographic photometry. For the variable star work in the Large Cloud we have used secondary and tertiary sequences, referred to Sequence V. For the work on the constellations we used the new photoelectric standards directly; the sequence is located in the middle of the area of operation and its red and blue magnitudes extend over the interval needed for constellation star colors.

The requisite blue and red plates have been made with the Armagh-Dunsink-Harvard telescope for which the scale and speed are adequate to resolve the constellations and reveal the nebulosity associated with them. The newer fast emulsions, sensitive to the red and infra-red, have facilitated the work. The red magnitudes of the sequence were derived by applying the factor of 1.4 to the yellow-minus-blue color indices of van Wijk.

2. The five constellations selected for examination all contain stellar groups that bear NGC numbers. In the following listing (table 1) one NGC number identifies the position. Constellation I is a double aggregation, and III is a triple. Constellation V lies in the bar of the Large Cloud and the others are on the north side. Measured areas in each region are square, varying from $17' \times 17'$ to $33' \times 33'$, but the groupings that we identify as constellations are all elongated or irregular in outline.

Although only 230 of the supergiant stars are assigned to constellation membership, we have measured the photographic magnitudes of approximately 900 stars brighter than 14.0 in the red (complete to 13.8), and determined the colors for 490. The adopted magnitudes, both blue and

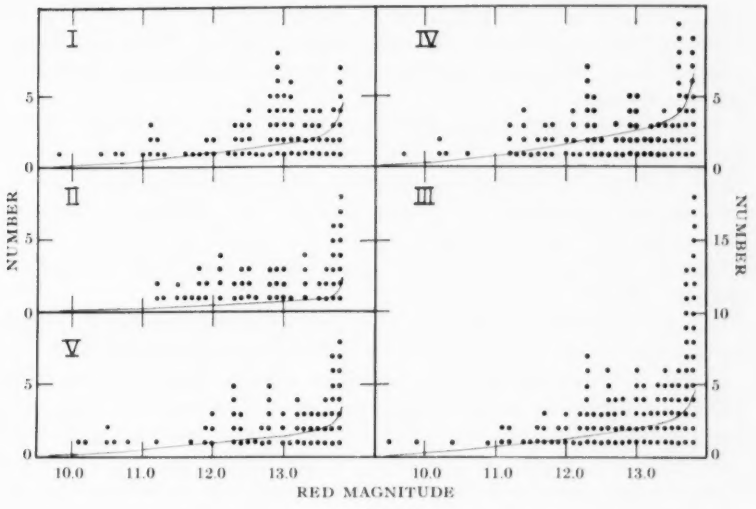

FIGURE 1

red, are each the means of two measures. The individual values are not published at this time since later most of these stars will be more accurately measured with photoelectric photometers. In order to get an idea of an average area that is free of constellation grouping, we have studied a control field, $50' \times 40'$, which is identical with the field of Sequence V. We estimate from the van Rhijn tables of star density (Groningen 43) that about half the stars in the control region are actually in the (galactic) foreground. The Large Cloud is so irregular in structure and in the distribution of its supergiant stars that the elimination of background magnitudes and colors for the individual constellations is quite uncertain. Nevertheless we indicate an average background and foreground in the

plots of figure 1. There is also, in all of the fields, a sparse scattering of objects brighter than photographic magnitude 10.5 (too bright for measuring on the ADH plates), which we believe, on the basis of colors and frequency, to be foreground galactic stars.[3]

3. In figure 1, the frequency of red magnitudes is shown for all five fields. The full line drawn through each distribution plot shows the magnitude distribution in the control field, reduced for each field to the appropriate area. The distribution of the constellation supergiants is shown by the points above the curves. To see what fainter stars could be properly allocated to the constellations, we have also counted for each area the total number of stars on a blue plate to photographic magnitude 15.2. The populations continue to stand far above that of the control field. Giant blue stars like those in Scorpius and Orion appear to be abundantly associated with the supergiants in all five of the Magellanic constellations. The total populations of these aggregations probably run into the thousands.

On the basis of the present revision of the distance of the Large Cloud, the modulus is about 18.6 (uncorrected, as are the constellation magnitudes, for space absorption), and therefore the red absolute magnitudes of stars represented in figure 1 range from -5 to -8. They are exceeded in luminosity very rarely throughout the Cloud. S Doradus, which lies in Constellation V, has an absolute photographic magnitude of approximately -10.

4. The distribution of the red color indices is shown in figure 2. Again in this figure a curve represents color distribution in the control field.

Associated with the many constellation stars of negative color index are a few that are as red as the local supergiants, Antares and Betelgeuse, but probably much brighter. The last column of table 1 suggests the approximate numbers. If they are truly members of the Cloud, their low surface brightness indicates colossal dimensions, such as are found for the red variable stars of three types, which were discussed at the American Astronomical Society Meeting in June, 1951.[4] The measured colors are in general agreement with the spectra classified by Miss Cannon and published in the Henry Draper Extension.[5]

The spread of red indices is very large. Many are bluer than $-0^m.5$, thus indicating that very little space absorption exists between us and these stars in the Large Cloud. The galactic latitude is $-33°$, and we have therefore adopted as the total photographic absorption $+0^m.3$. The Large Cloud is in a field as rich in faint background galaxies as are the galactic polar caps. The small space absorption supports, incidentally, the essential correctness of van Wijk's photoelectric sequences, which have also been independently confirmed by the photoelectric work of Gascoigne and Kron.[6]

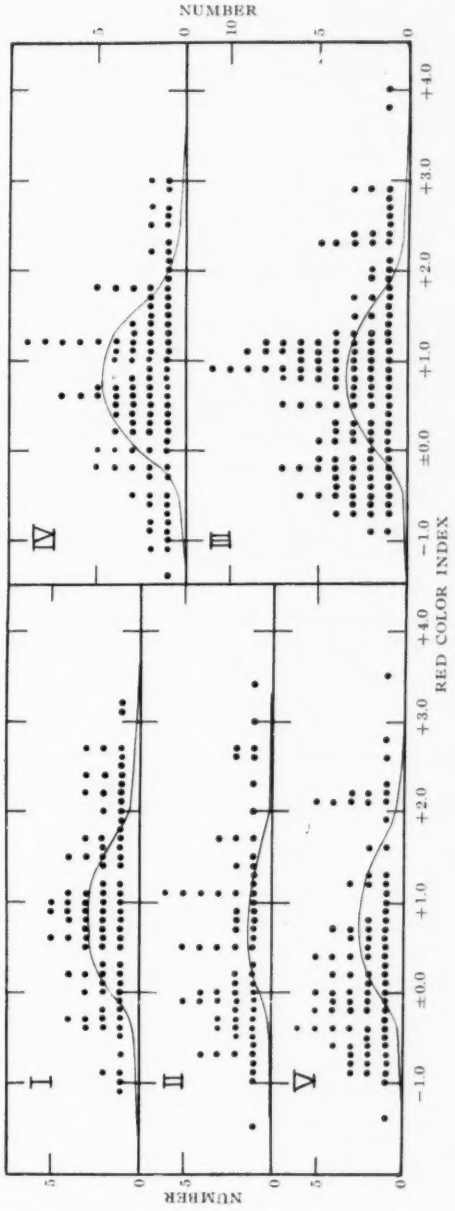

FIGURE 2

A few bright variables with large color indices are within the areas measured for constellations. They are practically all certain members of the Large Cloud, and in luminosities and linear dimensions must be comparable with those reported in our earlier note on the superluminous variables.[4] Of more interest are the extremely red stars which have been detected in the course of this work through the use of Eastman I-N infrared plates. They will be reported in some detail when a survey of stars of this sort throughout the Cloud is published; but here we note that such objects appear mostly in parts of the constellations not affected by conspicuous nebulosity. They may be younger than any other stars in the Cloud.

5. *Summary.*—We have examined five stellar aggregations in the Large Magellanic Cloud that average something more than one thousand light years in diameter. We call them constellations because of their rough similarity in size, population, and star colors to the Orion constellation and similar stellar associations in our galactic system. The luminosities of the blue member stars range from -8 to -5 in absolute photographic magnitude. A few exceedingly red supergiant stars also appear in these constellations, as well as a large number of blue stars fainter than $M = -5$, as in the Orion,[7] Perseus,[8] and Scorpius-Centaurus[9] groups.

[1] Formerly called sub-Clouds (*Harv. Obs. Bull.* 884 (1931)) and perhaps remotely analogous to what Ambartsumian would call "associations," but here restricted to supergiant stars, *Publ. Mich. Obs.*, **10**, 83 (1951).

[2] *Harv. Obs. Bull.* 921 (1952).

[3] Jennings, A. L., *Harv. Circ.* 438 (1940).

[4] Abstract, *Ast. J.*, **56**, 139–140 (1951).

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[8] Blaauw, A., *B. A. N.*, **11**, 405–413 (1952).

[9] Blaauw, A., *Ibid.*, **11**, 413–433 (1952).

AROMATIC BIOSYNTHESIS. VI. RESISTANCE TO INHIBITION BY AN ACCUMULATED METABOLITE, 5-DEHYDROSHIKIMIC ACID, AND ITS SIGNIFICANCE FOR THE MECHANISM OF DRUG RESISTANCE*

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In *Escherichia coli* and other bacterial species 5-dehydroshikimic acid (DHS) and shikimic acid are successive precursors of a group of aromatic metabolites (tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid).[1] While many mutants that require these five compounds can utilize shikimic acid to replace them all, mutants blocked immediately before shikimic acid cannot do so. The reason for this failure is that these strains accumulate DHS and this compound inhibits competitively the utilization of shikimic acid.[2]

These DHS-inhibited mutants, however, give rise to secondary mutants that can grow on a minimal medium supplemented with shikimic acid. Among these, three types have been recognized.[2] One is a reversion which can grow without any supplement. The second retains the original block and has an additional block, before DHS, which prevents the accumulation of this inhibitory substance and thereby releases the ability to grow on shikimic acid. The third type also retains the quintuple requirement, and has gained the ability to grow on shikimic acid despite the fact that it apparently still accumulates DHS.

This paper will show that this third type of mutant accumulates DHS as extensively as its parent strain, and therefore must have become resistant to inhibition by this compound. The significance of this phenomenon for the mechanism of drug resistance will be discussed.

Methods.—The minimal medium used has been described.[3] Quantitative assays for DHS were performed turbidimetrically with *E. coli* mutant strain 83-1, using the minimal medium supplemented with 20 μg. of L-tyrosine and 40 μg. of DL-phenylalanine per ml.[1]

Results.—Strain 156-53 is a quintuple aromatic auxotroph of *E. coli* that accumulates DHS and cannot grow on shikimic acid. The isolation from this strain of secondary mutants that can grow on shikimic acid has been described elsewhere.[2] Table 1 presents results of a typical accumulation experiment performed with this parent strain and two of these secondary mutants. It is seen that strain 156-53D2, which has been shown to be doubly blocked,[2] does not accumulate DHS. In contrast, the other secondary mutant (156-53R11) accumulates as much as the

parent strain. (In this experiment strain 156-53R11 actually accumulated more than its parent, but the difference is not significant for the purpose of the present analysis.)

The identification of the DHS accumulated by strains 156-53 and 156-53R11 was based on several criteria:[1,4] growth factor activity for mutants blocked before DHS but not for those blocked after it; chromato-

TABLE 1

ACCUMULATION OF 5-DEHYDROSHIKIMIC ACID BY VARIOUS MUTANTS

A loopful of a turbid suspension of each strain was inoculated into 10 ml. of minimal medium containing 0.5% glucose and supplemented with L-tyrosine 10, DL-phenylalanine 20, L-trytophan 5, *p*-aminobenzoic acid 0.01, and *p*-hydroxybenzoic acid 0.01 μg./ml. The cultures were incubated with shaking at 37°C. Assays were performed on filter-sterilized samples.

STRAIN	TYPE	5-DEHYDROSHIKIMIC ACID (μG./ML.) 24 HOURS	48 HOURS
156-53	Parent	1	60
156-53R11	Resistant	1	100
156-53D2	Double block	0 (<0.5)	0 (<0.5)

TABLE 2

GROWTH RESPONSE OF VARIOUS MUTANTS TO SHIKIMIC ACID

Fresh overnight cultures of the three mutants in a medium supplemented with 0.2% NZ-Case (tryptic digest of casein) and 0.2% Difco yeast autolystate were washed with phosphate buffer and resuspended in buffer to an equal turbidity. Inocula of 0.1 ml. were added to 10 ml. of minimal medium, supplemented as indicated, in 22 mm. colorimeter tubes. These were then incubated at 35°C and read at intervals in an Evelyn photoelectric colorimeter, 620 mμ filter.

SUPPLEMENT (μG./ML.)		TIME (HOURS)	STRAIN 156-53	STRAIN 156-53R11	STRAIN 156-53D2
			OPTICAL DENSITY		
Shikimic acid	30	0	0.01	0.01	0.01
		5	0.01	0.01	0.01
		7	0.01	0.03	0.01
		8.5	0.01	0.06	0.02
		10	0.01	0.09	0.04
		11.5	0.01	0.14	0.07
		24	0.02	0.22	0.24
None		24	0.01	0.01	0.01

graphic mobility; instability to alkali; quenching of ultra-violet fluorescence on paper chromatograms; and inhibition of the response of doubly blocked mutant 156-53D2 to shikimic acid.

Strains 156-53 and 156-53R11 showed a quantitatively identical quintuple aromatic requirement. The only detectable difference between these strains was found in their response to shikimic acid. Table 2 shows that in a liquid medium with shikimic acid as sole supplement strain 156-53 does not grow perceptibly, whereas strain 156-53R11 grows even more

rapidly than doubly blocked mutant 156-53D2.[5] Similar results were obtained on solid media. It is therefore concluded that the secondary mutation in 156-53R11 has made it resistant to inhibition by the DHS that it continues to accumulate.

Discussion.—Competitive inhibition by normal metabolites is of particular interest because of its possible regulatory function in metabolism.[6, 7] The present results show that a mutation resulting in loss of such an inhibition does not necessarily involve a quantitative change in the concentration of the inhibitory metabolite or of its antagonist.

This example of resistance to an endogenous inhibitor is a special case of the general phenomenon of genetically acquired resistance to a growth inhibitor (i.e., "drug resistance"). The present observations are therefore of further interest because of their implications for the biochemical mechanism underlying this phenomenon.

A number of theoretically possible mechanisms have been listed elsewhere.[8] Several of these can be ruled out for the DHS-resistant strain because of the fact that the circumstances usually encountered in competitive inhibition are here reversed: the inhibitor is supplied endogenously and its reversing metabolite exogenously. Thus (1) decreased penetration of the inhibitor can be definitely excluded since this compound arises within the cell and appears in undiminished amount. The same argument excludes (2) increased destruction of the inhibitor. Similarly, resistance cannot be based on (3) increased synthesis of the inhibition-reversing metabolite since this metabolite cannot be synthesized at all.

Another mechanism (4), a decreased quantitative requirement for the product of the inhibited reaction, is conceivable where catalytic metabolites are involved, but seems very unlikely for a precursor that is converted mostly to building blocks (aromatic amino acids). In addition, shikimic acid is efficiently used (i.e., in approximately equimolar amounts) to replace aromatic metabolites in auxotrophs lacking the mutation to DHS resistance,[1] and this mutation does not detectably change the requirement for these metabolites or for shikimic acid. These results, of course, do not exclude the possibility that other drug-resistant mutants may involve one or another of these four mechanisms.

The properties of the DHS-resistant strain are compatible with several other mechanisms of resistance: (5) an alternative metabolic pathway bypassing the DHS-inhibited reaction; (6) an increased concentration of the inhibited enzyme; and (7) the formation of an enzyme with decreased relative affinity for the inhibitor compared with the metabolite. Among these, the development of an alternative pathway seems most unlikely in view of the lack of precedent for such an extensive effect of a single mutation; furthermore, DHS appears to compete directly with shikimic acid,[2] and this compound is not by-passed in the resistant mutant. Mechanisms

6 and 7 cannot be investigated further at present since the reaction of which shikimic acid is substrate is not known. It might be noted that evidence for the formation of an enzyme of altered affinity (mechanism 7) has been obtained with certain other drug-resistant mutants.[8]

Summary.—It has been shown previously that 5-dehydroshikimic acid is accumulated by certain bacterial mutants and interferes competitively with their growth response to shikimic acid.

From such a strain a secondary mutant has been isolated which is resistant to inhibition by the 5-dehydroshikimic acid that it continues to accumulate. Since the usual conditions of competitive inhibition are here reversed, the inhibitor being endogenous and the reversing metabolite exogenous, it is possible in this case to eliminate several of the mechanisms that have been invoked to explain the analogous phenomenon of drug resistance: decreased penetration of the inhibitor, increased destruction of it, and increased synthesis of the reversing metabolite.

Acknowledgment.—It is a pleasure to acknowledge the excellent technical assistance of Elizabeth S. Mingioli.

* Aided by a grant from the Rockefeller Foundation.

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[2] Davis, B.D., *Ibid.*, **64,** 749 (1952).

[3] Davis, B. D., and E. S. Mingioli, *Ibid.*, **60,** 17 (1950).

[4] Salamon, I. I., and B. D. Davis. Unpublished observations.

[5] The slightly slow growth of the doubly blocked mutant is very likely due to a secondary effect of its additional block before DHS. Evidence has been presented elsewhere[2] that interference with shikimic acid utilization is shown by mutants with a single block before DHS, though this effect is less marked than that resulting from a block immediately after DHS.

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*THE EFFECT OF ULTRAVIOLET IRRADIATION ON CELL DIVISION AND ELONGATION IN TIMOTHY ROOTS**

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Communicated by E. W. Sinnott, March 2, 1953

Cell size in growing organs and tissues depends on a number of factors, among which are the rate of cell expansion, the frequency of division into smaller cells, and the extent of expansion after the cessation of division. The relationship between these factors can be readily observed in the primary roots of the small seeded grasses where the division and elonga-

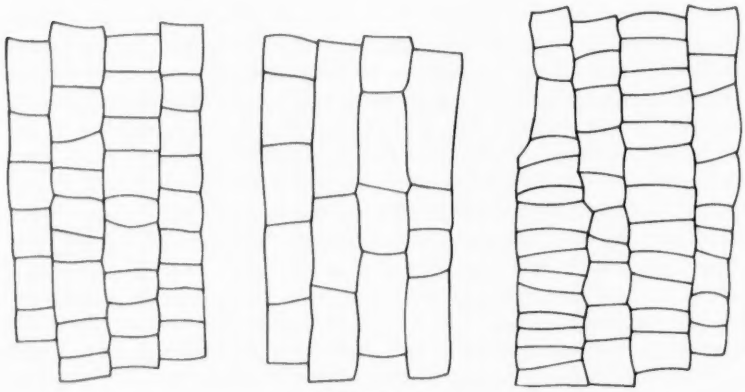
tion of individual surface cells can be seen directly. The roots are essentially cylindrical, and during growth of the root most of the expansion of its cells is in a direction parallel to the root axis. Thus the rate of increase in length of the cell is a fairly reliable measure of its rate of expansion. In the meristematic parts of the root most of the new cell walls appear at right angles to the long axis of the root, dividing the cells into shorter units. The process of division apparently does not influence the rate of elongation of the cell which is rather uniform throughout the meristematic zone.

Under constant environmental conditions, the pattern of cell size in the growing point remains essentially the same for a period of 3–4 days after germination.[1] Any agent which affects differentially the rate of elongation of the cells and their frequency of division will obviously alter the normal pattern of cell length. In the course of an investigation on the effects of ultra-violet radiation on seedling roots of timothy (*Phleum pratense*), it was observed that 10–12 hours after the unilateral irradiation of the root, cells in a few rows of the surface layer were unusually long, while the cells in adjacent rows were of normal length. It is well known that ultra-violet inhibits cell division in many organisms.[2] Thus it appears possible that ultra-violet differentially affects cell division and elongation, cell division being inhibited in the cell rows on the irradiated side while their rate of elongation remains the same as adjacent cells unaffected by the radiation. Such a differential effect shows the direct relationship between elongation and division in determining cell size, and is of sufficient interest to students of morphogenesis to warrant this preliminary report.

Timothy seedlings can be grown on the inner surface of a cover slip which forms one side of a small moist chamber. The root adheres to the cover slip as it grows downward and its surface cells can be observed directly. Photomicrographs taken at successive intervals give a permanent record of the rates of elongation and division of the surface cells in the growing point of the root. The construction of the moist chamber and method of taking the photographs have been described in detail elsewhere.[3]

The root sometimes tends to twist about its central axis as it grows downward, the twisting being especially pronounced in roots subjected to unilateral irradiation. This twisting may be sufficient to make it impossible to follow the same row of cells, so that rows of cells affected by unilateral irradiation of the root cannot be observed over prolonged periods of time. In order that irradiated cells might always be presented to view, even though twisting should occur, the root was rotated during irradiation so as to expose all sides equally. This was accomplished by inserting the seedling, previously germinated on moist filter paper until the root was

5–7 mm. in length, into a small glass tube with the root protruding from the end of the tube, the seedling being held in place by moist cotton. The glass tube was then inserted into a rubber stopper and the stopper fitted into a small quartz flask, the length of the tube being so adjusted as to suspend the root in the center of the flask. A few drops of distilled water were put into the flask to keep its sides wet, thus maintaining a high humidity. The flask and the contained seedling was turned at about ten revolutions per minute during exposure. The ultra-violet source was a 15-watt General Electric germicidal lamp which is said to deliver more than 80% of its energy in the 2537 Å region. Exposures were made at a



FIGURES 1–3

Changing cell size pattern in timothy root meristems following irradiation with ultra-violet.

1. Traced from photographs taken 10 minutes after exposure. The distal cells are at the bottom of the figure and about 0.07 mm. behind the tip.
2. Eleven hours after irradiation. Same distance from tip as figure 1.
3. Twenty-six hours after irradiation. Same distance as figure 1.

distance of 50 cm. from the root for 6 minutes at an intensity of about 8 ergs/mm.^{2}/second. Immediately following irradiation the seedling was transferred to a moist chamber for observation. Photographs of the growing point were taken at one-hour intervals from 8:30 A.M. through 9:30 P.M., and from 8:30 A.M. through 2:30 P.M. the following day. The experiments were carried out at room temperature, about 27°C.

Figure 1 shows the outlines of a group of surface cells in the meristematic zone traced from a photograph taken about 10 minutes after irradiation. The apical cells (bottom of the figure) were about 0.07 mm. behind the root apex (root cap not included). The total length of the 33 cells shown is 450 μ, or about 13.6 μ per cell. A group of cell outlines traced from a

photograph of the same root taken $10^1/_2$ hours after irradiation is shown in figure 2, the cell group being the same distance from the root apex as that shown in figure 1. The lengths of the 16 cells total 445 μ or about 27.8 μ per cell, approximately twice the length of the meristematic cells 10 minutes after irradiation. Figure 3 shows a group of cell outlines, from the same root and the same distance behind the root apex, 26 hours after irradiation. The total length is 485 μ for the 46 cells or about 10.5 μ per cell. Evidently, exposure to ultra-violet resulted in a temporary increase in the length of the surface cells in the meristematic zone. This may have been due either to a stimulation of cell elongation or to a decrease in frequency of cell division.

During the period from 10 minutes to $10^1/_2$ hours after irradiation, the cells elongated at an exponential rate of about 5.3% per hour as determined by measurements of individual meristematic cells. This is the period in which cell length increased. During the interval from 24 to 30 hours after irradiation, the rate of cell elongation was about 11% per hour, but average cell length was approximately the same as that at the time of irradiation. It is evident that the increase in cell length in the earlier period did not result from a stimulation of cell elongation but from an inhibition of cell division. Though the rate of cell elongation prior to irradiation is not known it is probable that this dosage depressed cell elongation, since in untreated roots the rate is relatively constant, rather than increasing over a 30-hour period.

The temporary depression of division followed by mitotic activity during the night hours cannot be attributed to a rhythm of mitotic activity, low during the day and high at night, since non-irradiated material does not show a periodicity in either the length of meristematic cells or in the frequency of cell division. It was thought that the resumption of cell division might be due to photoreactivation by the exposure to light necessary for the photography. To test this possibility an experiment was run using four roots, two of which were kept in complete darkness after irradiation and two photographed as in the original experiment. Twenty-four hours after irradiation all four were photographed and cell lengths measured. Average cell length was 11.5 μ in roots kept in darkness and 13.1 μ for those exposed to light. This difference is no more than normal variation in different roots.

The effect of the dosage used on the frequency of cell division is apparently limited to the surface layer, since the cortical cells immediately below appeared, from direct observation, to be of normal length. Ultra-violet radiation is of such limited penetration that probably little of its energy is transmitted through the surface cells to the cortex. The root cap, though small, covers about 30–40 μ of the extreme tip of the meristem. The apparent recovery of the ability to divide is probably not actually a

recovery of individual cells, but rather a replacement of the affected meristematic cells by the descendants of unaffected ones protected by the root cap at the time of irradiation.

Each meristematic cell is progressively displaced from the root apex because of the elongation of cells between it and the apex. When the cell is about 150–200 μ behind the root apex it ceases to divide and its rate of elongation gradually increases to about 45–50% per hour. This is the "elongation" growth mentioned by some authors. During this phase the cell may increase in length by as much as twentyfold. In irradiated roots, the surface cells that do not form root hairs are, at the beginning of "elongation" growth, nearly twice as long as non-irradiated cells. Thus they elongate to *ca.* 400–500 μ as compared to the normal length of 200–250 μ. Evidently the surface cells which are affected by irradiation cannot always keep pace with the unaffected cortical cells below them, with the result that adjacent end walls of the surface cells are sometimes pulled apart (Fig. 4). This phenomenon has not been observed in untreated roots. The occurrence of such separations indicates that, in irradiated material at least, the surface cells may be largely passive in the growth of the root. There is good evidence that cell walls do not slide along one another in these roots.[4] In the absence of such sliding growth the surface cells must elongate at the same rate as the unaffected cortical cells below and may even act as a brake on the elongation of the cortical cells. Therefore, though it is evident that ultra-violet inhibits division in the surface cells, its effects on their elongation is not clear, since their elongation is influenced by the underlying cortical cells. These results show directly that the differentiation of large and small cells in multicellular organisms is not necessarily due to differences in the rates of expansion of different cells, but may be brought about by a differential frequency of division.

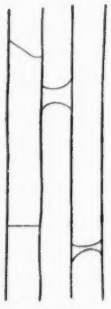


FIGURE 4

Separation of adjacent end walls of elongating surface cells in timothy roots following exposure to ultraviolet radiation.

Summary.—Irradiation of timothy roots with ultra-violet inhibits division of the surface cells although their elongation continues, but at a reduced rate. As a result the affected cells become enormously long and in some cases are unable to keep pace with the elongating unaffected cortical cells beneath, resulting in the separation of end walls of adjacent surface cells. It is shown directly, in the living root, that large cells may result from an inhibition of cell division rather than from a stimulation of cell expansion.

* Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

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ENZYMES, SPECIFIC SUBSTRATES AND COMPETITIVE INHIBITORS AS MULTIFUNCTIONAL ENTITIES

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In the development of the Michaelis-Menten theory of enzyme-catalyzed reactions[1,2] it has been repeatedly recognized that under certain conditions the catalytically active site of the enzyme may be capable of simultaneous interaction with two or more molecules of a particular specific substrate or competitive inhibitor and thus form a variety of ternary and possibly higher complexes.[3–9] This implication of the possible multifunctionality of a catalytically active site has led us to consider the more general situation wherein not only the catalytically active site is considered to be multifunctional but also the specific substrate or competitive inhibitor.[9–11]

TABLE 1

BINARY AND TERNARY COMPLEXES DERIVABLE FROM THE COMPLEMENTARY INTERACTION OF A BIFUNCTIONAL SPECIFIC SUBSTRATE WITH A BIFUNCTIONAL ACTIVE SITE

COMPLEX	INTERACTION	REACTANTS	CONSTANTS
ES_1	$(R_1 - \rho_1)$	E_f, S_f	K_{S_1}
ES_2	$(R_2 - \rho_2)$	E_f, S_f	K_{S_2}
ES_{12}	$(R_1 - \rho_1, R_2 - \rho_2)$	ES_1; ES_2; E_f, S_f	$K_{S_{12}}$; $K_{S_{21}}$; K_{ES}
ES_1S_2	$(R_1 - \rho_1)(R_2 - \rho_2)$	ES_1, S_f; ES_2, S_f; E_f, $2S_f$	$K_{S_1S_2}$; $K_{S_2S_1}$; K_{ESS}
E_1S_2E	$(R_1 - \rho_1)(R_2 - \rho_2)$	ES_1, E_f; ES_2, E_f; $2E_f$, S_f	$K_{S_1E_2}$; $K_{S_2E_1}$; K_{ESE}

For simplicity let us first consider the case where both the active site of the enzyme and the specific substrate are bifunctional, i.e., where the specific substrate can be considered to possess two distinguishable structural characteristics R_1 and R_2 and the active site of the enzyme two structural features ρ_1 and ρ_2 with which R_1 and R_2 can interact in a complementary manner. It will be assumed that all other types of interaction, i.e., $R_1 - \rho_2$, $R_2 - \rho_1$, $\rho_1 - \rho_1$, $\rho_2 - \rho_2$, $\rho_1 - \rho_2$, $R_1 - R_1$, etc., can be ignored.

In contrast to the classical presentation depicted in equation (1)

$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1_f} + P_{2_f} \ldots \qquad (1)$$

one can anticipate in the bi-bifunctional system in addition to E_f, the free enzyme, and S_f, the free specific substrate, the possible presence of all of the species listed in table 1. The equilibria involved may be conveniently classified as: unimolecular, i.e., $ES_1 \overset{K_{S_{12}}}{\rightleftharpoons} ES_{12} \overset{K_{S_{21}}}{\rightleftharpoons} ES_2$; bimolecular, i.e., $E_f + S_f \overset{K_{S_1}}{\rightleftharpoons} ES_1$, $E_f + S_f \overset{K_{S_2}}{\rightleftharpoons} ES_2$, $E_f + S_f \overset{K_{ES}}{\rightleftharpoons} ES_{12}$, $ES_1 + S_f \overset{K_{S_1S_2}}{\rightleftharpoons} ES_1S_2$, $ES_2 + S_f \overset{K_{S_2S_1}}{\rightleftharpoons} ES_1S_2$, $ES_1 + E_f \overset{K_{S_1E_2}}{\rightleftharpoons} E_1S_2E$, $ES_2 + E_f \overset{K_{S_2E_1}}{\rightleftharpoons} E_1S_2E$; and termolecular, i.e., $E_f + 2S_f \overset{K_{ESS}}{\rightleftharpoons} ES_1S_2$, and $2E_f + S_f \overset{K_{ESE}}{\rightleftharpoons} E_1S_2E$.

Again for simplicity it will be assumed that of all of the intermediate complexes only ES_{12} can be subsequently transformed into free enzyme and reaction products and that this reaction can be represented by equation (2). It is conceivable that under some circumstances the two ternary com-

$$ES_{12} \xrightarrow{k} E_f + P_{1_f} + P_{2_f} \ldots \qquad (2)$$

plexes ES_1S_2 and E_1S_2E could also serve as reactive intermediates and it is not implied that the transformation of ES_{12} into free enzyme and reaction products is necessarily an intramolecular process but rather that under the experimental conditions ordinarily encountered the specific rate constant k may be that of an apparent zero order reaction with respect to $[S]$.

For the condition that $[S_f] \doteq [S]$, i.e., where $[S] \gg ([ES_1] + [ES_2] + [ES_{12}] + [ES_1S_2] + [E_1S_2E])$ and where $d[ES_{12}]/dt \doteq 0$, i.e., where all equilibria are established rapidly relative to the rate $k[ES_{12}]$, and where E_f does not interact with any of the reaction products, i.e., for initial rates, we may, as a reasonable approximation, exclude the two termolecular processes described by the constants K_{ESS} and K_{ESE}, and specify the concentrations of the binary and ternary complexes arising from unimolecular or bimolecular processes as follows:

$$\begin{aligned}
[ES_1] &= [E_f]\,[S]/K_{S_1} = K_{S_{12}}[ES_{12}];\\
[ES_2] &= [E_f]\,[S]/K_{S_2} = K_{S_{21}}[ES_{12}];\\
[ES_{12}] &= [E_f]\,[S]/K_{S_1}K_{S_{12}} = [E_f]\,[S]/K_{S_2}K_{S_{21}} = [E_f]\,[S]/K_{ES};\\
[ES_1S_2] &= [ES_1]\,[S]/K_{S_1S_2} = [ES_2]\,[S]/K_{S_2S_1} = K_{S_{12}}[ES_{12}]\,[S]/K_{S_1S_2};\\
[E_1S_2E] &= [ES_1]\,[E_f]/K_{S_1E_2} = [ES_2]\,[E_f]/K_{S_2E_1} = K_{S_{12}}\,[ES_{12}]\,[E_f]/K_{S_1E_2}.
\end{aligned}$$

For the special condition where $k[ES_{12}]$ is directly proportional to $[E]$, i.e., the one ordinarily encountered in kinetic studies of isolated enzyme systems where $[S] \gg [E]$, we may ignore the contributions to the rate equation arising from the presence of E_1S_2E and arrive at equations (3) and (4).

$$[E_f] = [E] - \{(1 + K_{S_{12}} + K_{S_{21}}) + (K_{S_{12}}/K_{S_1S_2})\ [S]\}\ [ES_{12}]. \quad (3)$$

$$[ES_{12}] = [E]\ [S]/(K_{S_1}K_{S_{12}} + [S]\ (1 + K_{S_{12}} + K_{S_{21}}) + (K_{S_{12}}/K_{S_1S_2})\ [S]^2). \quad (4)$$

When $-d[S]/dt = v$, $A = K_{S_1}K_{S_{12}}$, $B = 1 + K_{S_{12}} + K_{S_{21}}$, and $C = K_{S_{12}}/K_{S_1S_2}$, the rate equation is

$$v = k[E]\ [S]/(A + B[S] + C[S]^2) \quad (5)$$

which may be divided by the quantity B/B to give equation (6) where $V = k[E]/B$.

$$v = V[S]/(A' + [S] + C'[S]^2). \quad (6)$$

Equation (6) is of the same general character as the rate equation for the classical treatment, i.e., equation (7), but in the latter instance $V = k_3[E]$.

$$v = V[S]/(K_S + [S]). \quad (7)$$

If observations are limited, by design or by chance, to the experimental situation where $(A + B[S]) \gg C[S]^2$ it follows[5] that a plot of $1/v_0$ vs. $1/[S]_0$ will be linear and will have an intercept of $1/V$ and a slope of $A'/V = K_S/V$. The fact that $V = k_3[E] = k[E]/B$ is of particular interest because it generally has been assumed that k_3, even though it is determined by an extrapolation procedure, is necessarily the specific rate constant for the transformation of ES into E_f and reaction products and is therefore independent of any equilibria involved in the formation of ES. It is now clear that the above assumption may not be valid in all cases, and that a change in V associated with a change in the molecular structure of a specific substrate, or of the enzyme, may not be due to only a change in k, but may also be due, either wholly or in part, to a change in the values of one or more of the equilibrium constants of the various intermediate enzyme-substrate complexes.

Rate equation (6) predicts inhibition by the specific substrate and the relation existing between v and $[S]$ for all values of $[S]$ can be calculated from this equation. V and A' are evaluated as described above and C' is evaluated from a determination of v when $C'[S]^2 \gg (A' + [S])$ by a plot of v_0 vs. $1/S_0$ based upon equation (8). It follows from equation (6) that the maximum velocity is attained when $[S] = \sqrt{A'/C'}$.

$$v = V/C'[S]. \quad (8)$$

The above treatment of the interaction of a bifunctional catalytically active site with a bifunctional specific substrate has already found application in an interpretation of the effect of varying concentrations of auxins upon the growth of Avena coleoptile sections.[12]

If, in a system containing an enzyme with a bifunctional catalytically

active site and a bifunctional specific substrate, there is introduced a monofunctional competitive inhibitor I, e.g., a compound which will interact with the active site only via a $R_1 - \rho_1$ interaction we will have in addition to the complexes listed in table 1 those arising from the following equilibria:

$$[EI_1] = [E_f]\,[I_f]/K_{I_1}$$
$$[ES_2I_1] = [ES_2]\,[I_f]/K_{S_2I_1} = [EI_1]\,[S_f]/K_{I_1S_2}.$$

The rate equation for this situation is given by

$$v = k[E]\,[S]/\{A(1 + [I]/K_{I_1}) + (1 + K_{S_{12}} + K_{S_{11}}(1 + [I]/K_{S_2I_1}))\,[S] + C[S]^2\} \quad (9)$$

which can be simplified by the substitution $B_I = 1 + K_{S_{12}} + K_{S_{11}}(1 + [I]/K_{S_2I_1})$ to give

$$v = (k[E]/B_I)\,[S]/\{(BA'/B_I)(1 + [I]/K_{I_1}) + [S] + (BC'/B_I)\,[S]^2\}. \quad (10)$$

It can be seen from equations (6) and (10) that if $V_0 = k[E]/B$ in the absence of the inhibitor and $V_I = k[E]/B_I$ in the presence of the inhibitor, $V_I = (B/B_I)V_0$ and K_{I_1} and the ratio B/B_I can be evaluated by comparison of the slopes and intercepts of e.g., $1/v_0$ vs. $1/[S]_0$ plots with and without added inhibitor where the slope in the absence of the inhibitor is A'/V_0 and in the presence of the inhibitor $A'(1 + [I]/K_{I_1})/V_0$ and the intercept in the absence of the inhibitor is $1/V_0$ and in the presence of the inhibitor $1/V_0(B/B_I)$. Thus in contrast to the classical treatment[1-8] which predicts that the addition of a competitive inhibitor to a system containing enzyme and specific substrate will cause, in the above plot, only an increase in the slope and no change in the value of V, the present treatment predicts not only a change in the slope, but also a change in the value of V in the presence of the inhibitor from that in the absence of the inhibitor by an amount equal to B/B_I. There are at least three factors that may be responsible for the present lack of experimental confirmation of this prediction: (1) the relatively low order of precision found in most enzymatic studies; (2) the subjective nature of the methods ordinarily used in the construction of the plots; and (3) the fact that one of the several possible interactions may be so dominant that the value of B/B_I is equal to unity to within the limits of experimental error. In any event it is apparent that any perturbation of the various equilibria controlling the concentration of the reactive enzyme-substrate complex or complexes, whether produced by alternate modes of combination of the specific substrate with the enzyme or by an added competitive inhibitor, will influence to some degree the value of the so-called specific rate constant when the latter value, i.e., k_3, is determined by an extrapolation procedure.

For a system containing a bifunctional catalytically active site, a bi-

functional specific substrate and a bifunctional competitive inhibitor, the following additional equilibria must be considered:

$$\begin{aligned}
[EI_2] &= [E_f]\,[I_f]/K_{I_2} \\
[ES_1I_2] &= [ES_1]\,[I_f]/K_{S_1I_2} = [EI_2]\,[S_f]/K_{I_2S_1} \\
[EI_{12}] &= [EI_1]/K_{I_{12}} = [EI_2]/K_{I_{21}} = [E_f]\,[I_f]/K_{EI} \\
[EI_1I_2] &= [EI_1]\,[I_f]/K_{I_1I_2} = [EI_2]\,[I_f]/K_{I_2I_1}.
\end{aligned}$$

The rate equation for this situation is given by

$$v = (B/B_{I_{12}})V_0\,[S]/\{(B/B_{I_{12}})\,A'(1 + [I]/K_i + [I]^2/K_{I_1}K_{I_1I_2}) + [S] + (B/B_{I_{12}})C'[S]^2\} \quad (11)$$

where $B_{I_{12}} = 1 + K_{S_{12}}(1 + [I]/K_{S_1I_2}) + K_{S_{21}}(1 + [I]/K_{S_2I_1})$ and $1/K_i = 1/K_{I_1} + 1/K_{I_2} + 1/K_{I_1}K_{I_{12}} + 1/K_{I_2}K_{I_{21}}$. The character of this equation is such that an approximate graphical solution should give values for K_i only at low values of $[I]$. However it is again seen that the specific rate constant will be influenced by the concentration of the inhibitor and the various equilibrium constants.

Present circumstances do not justify the presentation of a general treatment of the case of the trifunctional catalytically active site interacting with a tri- or bifunctional specific substrate since in the former instance the simplest treatment involves a minimum of 14 intermediate complexes and the possibility of 4 reactive complexes. It may be noted that restricted cases involving the interaction of a trifunction catalytically active site with tri- and bifunctional specific substrates have been considered previously.[9-11]

It is believed that the most important conclusion that has been developed from the treatment given in this communication is that the so-called specific rate constants as ordinarily determined, i.e., the k_3 values, may be dependent upon the various equilibria involved in the formation of the reactive intermediates and that changes in the values of k_3 that are associated with changes in the molecular structure of specific substrates are not necessarily a reflection of changes in the susceptibility of ES to subsequent reaction but may be caused either wholly or in part by changes in the equilibrium concentrations of ES.

* Contribution No. 1790.

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*PAPER CHROMATOGRAPHIC PATTERNS OF GENETICALLY DIFFERENT TISSUES: A CONTRIBUTION TO THE BIOCHEMICAL STUDY OF INDIVIDUALITY**

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The discovery of differences related to descent, and the description in chemical terms of how a known genotypic condition present in the zygote may determine, or be related to, a definite morphology of the adult organism, are two of the aims of genetics. Paper partition chromatography of fresh tissues or body fluids in animal and plants provides a simple tool for the pursuit of these aims. In this report data will be presented showing how this technique, an outgrowth of the work initiated by Hadorn and Mitchell,[1] makes it possible to recognize genotypic differences hidden under identical morphological features; indications will be given, too, as to the range of applicability of the procedure used. Much remains to be studied yet, especially at the biochemical level, on the very material here presented. It seems worth while, however, at the present stage of these investigations, to summarize some of the results obtained. It is hoped that this might stimulate similar work on the same or other organisms and especially attract the interest and the cooperation of the biochemists.

Chromatographic Technique.—Both general procedures of chromatographic separation, by descending flow and by capillary ascent of the solvent, have been used in most cases. As the former allows a better separation of the spots than the latter, if not otherwise stated all the data and photographs reported have been obtained from chromatograms developed by descending flow, following standard techniques (see, e.g., Balston and Talbot[2]). For ascending chromatograms the technique of Williams and Kirby,[3] as modified by Hadorn and Mitchell,[1] has been used.

Sheets of Whatman No. 1 filter paper of various sizes were prepared as follows. In case of the descending chromatograms a light pencil line was drawn 3 in. from the edge to be dipped into the solvent, and samples for chromatography were placed at 1 in. or 4-cm. intervals along this line. In case of the ascending chromatograms the pencil line was drawn 1.5 cm. from the edge and samples applied at 1-cm. intervals. Details about the treatment of animal and plant tissues and fluids will be given below.

Several solvents as described by Kirby Berry, *et al.*,[4] have been tried; most of the descending chromatograms, however, have been obtained using either of the following mixtures.

(A) 2 parts (by volume) of *n*-propanol and 1 part of 1% ammonia.

(B) 4 parts of *n*-butanol, 5 parts of distilled water, 1 part of glacial acetic acid are poured into a separating funnel in the given order, and thoroughly shaken for about three minutes; separation of the two phases is allowed for at least two hours, at room temperature. Sheets for chromatography are hung in the tank, letting the vapours of the lower phase impregnate the paper for about one hour; the upper phase is then poured into the troughs.

Mixture (A) does not need to be renewed at each run, but (B) must be freshly prepared for each determination. For ascending chromatograms mixture (A) has been used. Solvent A will be indicated as 2P:1A; solvent B as B:A.

For descending determinations Chromatocabs Model 300 of University Apparatus Co., Berkeley, California, and for ascending runs glass jars of various sizes have been used. Time of development (or length of runs) has varied between 2 and 24 hours at 22°–25°C. Most of the reported determinations were obtained from 22-hr. runs; in this time the front of the solvent covers almost to the edge the length of Whatman No. 1 sheets. At the end of the period the sheets were removed from the tanks or jars and allowed to dry in the air at room temperature. Known mixtures of amino-acids have been constantly developed for control, together with the materials to be analyzed. Two groups of separating substances have been studied: fluorescent materials as revealed by use of a G.E. ultra-violet lamp (Black-Light Lamps B-H 4); ninhydrin-positive materials as revealed by spraying the developed chromatograms with a 0.2% solution of ninhydrin in 95% ethanol, to which 5% of 2,4,6-collidine was added before use. While the fluorescing substances may last unaltered for several weeks or months, the ninhydrin-positive materials fade out quickly, in a few days. Recording of the data and photographs of the chromatograms were correspondingly taken about 24 hr. after the ninhydrin spraying. Since no attempt was made toward a qualitative or quantitative identification of the chemical nature of the separating spots, the best type of record proved to be the direct photography of the fluorescent or ninhydrin-positive patterns. Black-and-white as well as colored photographs were taken and kept as permanent records. Cross checking of the constancy of the results were made by comparing (*a*) one-dimensional chromatograms obtained from the same genetic material at different times, (*b*) one-dimensional chromatograms developed for different times with the same solvent, (*c*) two-dimensional chromatograms with the one-dimensional ones obtained with the same solvents. Every statement concerning the presented material is

based on the analysis of a minimum of three repetitions, carried out independently in different days, on at least three individuals of each genotype; in each experiment a minimum of ten samples per genotype were developed.

DROSOPHILA MELANOGASTER

Culture Conditions.—The medium for growing flies had the following composition: molasses, 237 ml.; corn meal, 473 g.; brewer's dry yeast, 40 g.; agar, 30 g.; water, 2840 ml. After cooking and bottling the medium was seeded with Fleischmann's bakers' yeast or with a pure strain of *Saccharomyces cerevisiae*. In order to avoid crowding and to insure thereby constancy in the flies size, batches of about 100 eggs were collected and transferred to half pint bottles. Development took place at 25 ± 1°C. Flies which had undergone development on medium occasionally infected by green molds were not discarded but analyzed separately in order to check the influence of different diets. The observation made by Hadorn and Mitchell[1] that substances from the food contained in the gut of either larvae or flies are not present in sufficient quantities to interfere with any of the determinations were confirmed in the course of these experiments.

Preparation of the Flies.—Observations to be reported in this paper relate only to the chromatographic patterns of adult flies of various ages. Only morphological mutants not affecting eye pigment production were studied and, in order to get rid as much as possible of the pigment component in the chromatograms, flies have always been decapitated after etherization. These were then quickly washed in 95% ethanol and boiled for one minute in distilled water. After removing adhering water by placing them on filter paper, flies were transferred directly to the chromatograph sheets and squashed thoroughly onto the paper using a flat glass pestle. The spots so produced were allowed to dry in air at room temperature. Either single flies or three flies were mashed to form a single spot. Residual tissues were not removed. Once dry the sheets are storable for months and chromatograms can be developed at any convenient time; precautions against bacterial or mold contamination must be observed, of course.

Basic Pattern of the Adults and Its Constancy.—Comparison of the ninhydrin-positive patterns obtained from various Drosophila strains did not show any appreciable difference. The rest of this report concerning such material will be limited therefore to fluorescing substances. There is very little correspondence between the ninhydrin-positive and the fluorescing patterns. R_f values are given without standard errors since these would give the impression of a higher accuracy than one can actually obtain in such measurements; spots being often quite large, the actual measurement of the distance covered with respect to the front of the solvent is necessarily somewhat arbitrary. The given values, however, are the mean of at least 60 independent readings, and were mostly taken from 12 hr.

as well as from 20 hr. descending runs. As it will be shown, there is good agreement between values here reported and the ones published by Hadorn and Mitchell.[1]

By basic pattern it is meant the maximum number of distinguishable spots for any particular material and solvent. As will be shown below, variations in the patterns can always be related to this "basic pattern" in that the position and color of each spot does not change in different strains of this species. Differences between strains can refer to number and/or intensity, and/or size of the spots of the fluorescing materials. The particular combination of spots of any one strain gives a very characteristic pattern.

TABLE 1

R_f VALUES OF FLUORESCING SPOTS IN THE BASIC PATTERN OF DECAPITATED *Drosophila melanogaster*. SOLVENT: 2P:1A

FEMALES			MALES		
SPOT NO.	COLOR	R_f	SPOT NO.	COLOR	R_f
1	Yellow	0.088	1	Yellow	0.088
2	Blue	0.174	2	Blue	0.179
3	Green	0.215	3	Green	0.208
4	Greenish-blue	0.230	4	Greenish-blue	0.259
5	Bluish	0.342	5	Blue	0.346
6	Yellow	0.393	6	Blue	0.397
7	Bluish	0.462	7	Yellow	0.432
8	Blue	0.513	8	Blue	0.495
			9	Yellow	0.569

TABLE 2

R_f VALUES OF FLUORESCING SPOTS IN THE BASIC PATTERN OF DECAPITATED *Drosophila melanogaster*. SOLVENT: B:A

FEMALES			MALES		
SPOT NO.	COLOR	R_f	SPOT NO.	COLOR	R_f
1	Yellow	0.081	1	Bluish	0.078
2	Purplish-blue	0.157	2	Purplish-blue	0.145
3	Yellow	0.186	3	Greenish-orange	0.182
4	Bluish-yellow	0.254	4	Bluish-yellow	0.254
5	Blue	0.328	5	Blue	0.328
			6	Yellow	0.470

Females: 2P:1A: In the basic pattern of fluorescing materials separating from decapitated females one can distinguish eight spots. For description of the colors and R_f values see figure 1, and table 1.

B:A: In the basic pattern of decapitated females one can distinguish five spots. For description see figure 1 and table 2.

Males: 2P:1A: In the basic pattern of fluorescing materials separating from decapitated males one can distinguish nine spots. For description see figure 1 and table 1.

B:A In the basic pattern of decapitated males one can distinguish six spots. For description see figure 1 and table 2.

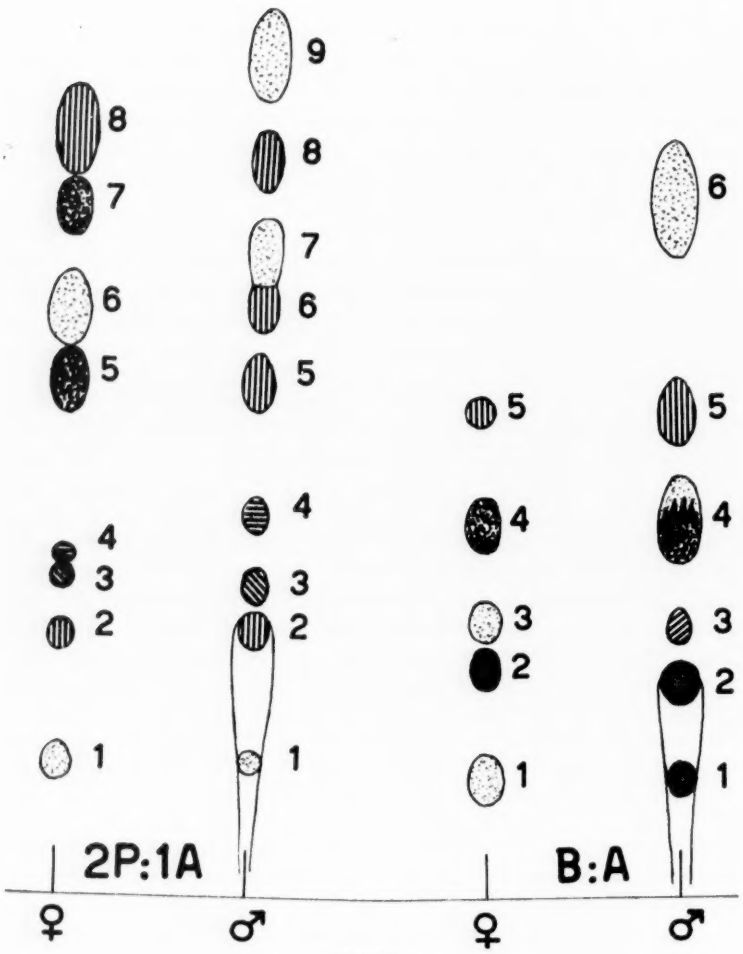

FIGURE 1

Schematic representation of the basic patterns of decapitated *Drosophila melanogaster*

With both solvents spot No. 2 in males makes a very bright blue stripe almost from the bottom of the chromatogram, which makes the difference with respect to females very obvious, and which often masks completely spot No. 1. This very striking difference between the fluorescing patterns of females and males is related to the presence of the blue fluorescing substance, which, according to Hadorn and Mitchell,[1] is especially concentrated in the testes, Malpighian tubes and haemolymph.

FIGURE 2

FIGURE 3

Fig. 2. Chromatographic patterns of fluorescing materials in *Drosophila melanogaster* females. Solvent: 2P:1A. Genotypes: *ss/ss;* homozygous wild type Samarkand; Samarkand/*ss;* homozygous wild type Oregon-R; Or/*ss*. Two chromatograms for each genotype are shown. Each chromatogram from three decapitated flies. Length of run: 20 hours.

Fig. 3. Chromatographic patterns of fluorescing materials in *Drosophila melanogaster* females. Solvent: 2P:1A. Genotypes: wild type Canton homozygous; cr^w/cr^w; *h/h; shv/shv; spa/spa; ss/ss; vg/vg.* Two chromatograms for each genotype are shown. Each chromatogram from three decapitated flies. Length of run: 20 hours.

The constancy of the observed patterns was studied with respect to two variable factors: feeding and age. As previously stated, different diets do not seem to influence appreciably the fluorescing patterns. In order to check the influence of age, samples of 200 eggs were collected for four strains (Canton, Samarkand, *shv, ss*) at five days intervals for a period of a

month. Embryonic and larval development as well as metamorphosis and hatching took place at 25°C.; at the twelfth day after egg collection, when all adults had hatched, flies were transferred to room temperature for aging. Correspondingly six groups of adults of the following ages from hatching were mashed onto the paper: ♀♀ 6–7 days; 10–11 days; 15–16 days; 21–22 days; 26–27 days; 31–32 days; ♂♂ 8–9 days; 12–13 days; 17–18 days; 23–24 days; 28–29 days; 33–34 days. A minimum of twelve chromatograms per age group and per strain was observed. While in the case of males age of the adults does not seem to influence at all the fluorescing pattern, every spot being quite constant over a period of about one month, in the case of females some effects of age were observed. Spot No. 2 (with both solvents) is very bright in early age but fades out and disappears almost completely after the twelfth day of adult life; spots No. 6 and 8 (2P:1A) are weaker in early age but reach the maximum of intensity and remain constant after the tenth day of life. As a consequence of these results, all the data reported below refer to animals aged at least for two weeks; in this way the age variable could be eliminated. In most cases two chromatographic patterns for each genotype are presented in the photographs in order to give an idea of the constancy of results.

Patterns of Wild Type Strains.—Four wild type strains were studied: Canton, Samarkand, Oregon-R, Florida 2436. A comparison of the photographs of three of these strains (see Figs. 2 and 6) will give an idea of the type of differences to be observed between strains. Oregon-R, e.g., has in females spots No. 3 and 8 much brighter than the other two; on the other hand Samarkand has the bluish No. 4 much more evident than either Canton or Oregon. The direct comparison of the chromatograms where the differences in color and shade of the fluorescing patterns can be appreciated or of their colored photographs allows a much easier distinction of the patterns of various genotypes. No attempt was made to evaluate quantitatively the differences in intensity and size of the spots. Among the strains available there was one which had been derived from the Oregon-R strain and had been inbred by brother-sister mating for 68 generations at the time when the material was used for chromatography. The pattern of fluorescing materials obtained from flies of this inbred strain was very similar to that of the Oregon strain. A comparison of a series of several dozens of chromatographic patterns obtained from single individuals of Oregon-R and of Oregon-R-inbred revealed that in the latter the individual variability was much smaller than in the former and that, correspondingly, the inbred strain had much more sharply clear-cut spots than the mass cultured strain from which it had been originally derived. It became possible, therefore, to evaluate the degree of genetic homogeneity by simple inspection of a series of individual chromatograms.

Patterns of Mutant Strains.—The different mutant strains tested are the

following: *Bx*, *bt*, *c*, *ci*^{W}, *en*, *ev*^{2}, *h*, *L*^{4}, *Ly*/*D*^{3}, *shv*, *spa*, *ss*, *ss*^{a}, *vg*, *ww*. For description of these mutants see Bridges and Brehme,[5] and D.I.S. 21.[6] Only those genotypes have been selected that do not affect appreciably the color of eyes or bodies. Mutants having different eye or body colors had previously been analyzed by Hadorn and Mitchell;[1] besides, it was thought of some interest to concentrate on mutants whose end-

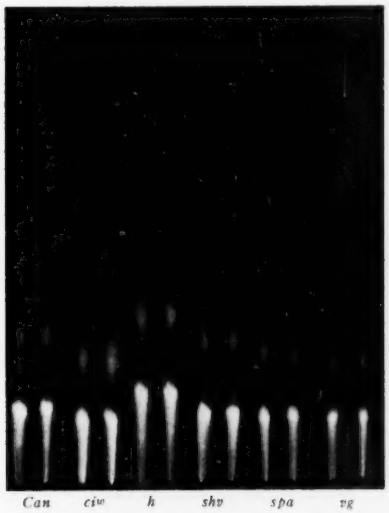

FIGURE 4

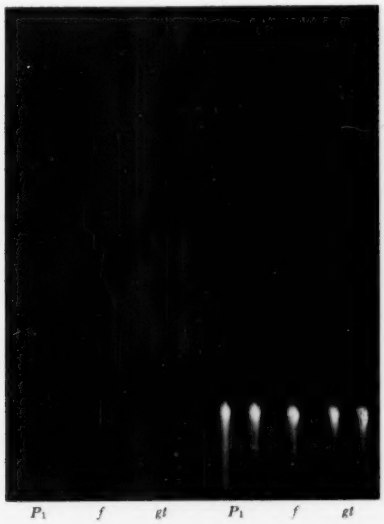

FIGURE 5

Fig. 4. Chromatographic patterns of fluorescing materials in *Drosophila melanogaster* males. Solvent: 2P:1A. The same genotypes as in Fig. 3. Two chromatograms for each genotype are shown. Each chromatogram from three decapitated flies. Length of run: 20 hours.

Fig. 5. Chromatographic patterns of fluorescing materials in *Drosophila melanogaster* females and males. Solvent: 2P:1A. Genotypes (from left to right): 2 chromatograms of homozygous wild type Oregon-R-S, ♀; 2 chromatograms of *f*/*f*, ♀; 2 chromatograms of *gt*/*gt*, ♀; 2 chromatograms of homozygous wild type Oregon-R-S, ♂; 1 chromatogram of *f*, ♂; 2 chromatograms of *gt*/*gt*, ♂. Each chromatogram from three decapitated flies. Length of run: 20 hours.

product, as observed at the phenotypic level, was not of an obvious chemical nature, such as pigment production, but rather affected a morphological structure, such as wing or bristle shape and size. Each of the tested strains proved to give a distinctive fluorescing pattern. Some of the differences are quite clear cut, e.g., one or more spots are entirely lacking, while some of the other differences are not so obvious, especially when considered in a black and white photograph. Figures 3 and 4 give, however, a picture of the type

of differences observed. As it will be noticed, a remarkable parallelism of results is to be seen when comparing the patterns of females and males of the same strain. A similar conclusion can be drawn with respect to the comparison of patterns derived from the same genotype but with different solvents. A detailed description of each pattern could be given in terms of number, intensity and size of the spots. This seems superfluous, how-

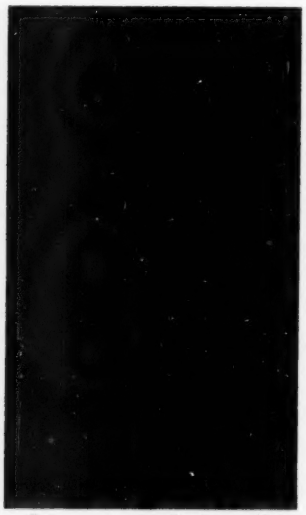

FIGURE 6

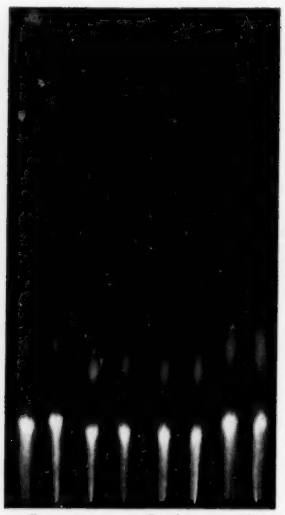

FIGURE 7

Fig. 6. Chromatographic patterns of fluorescing materials in *Drosophila melanogaster* females. Solvent: 2P:1A. Genotypes: homozygous wild type Canton; *ss/ss;* F_1 from ♀ Canton × ♂ *ss;* F_1 from ♀ *ss* × ♂ Canton. Two chromatograms for each genotype shown. Each chromatogram from three decapitated flies. Length of run: 20 hours.

Fig. 7. Chromatographic patterns of fluorescing materials in *Drosophila melanogaster* males. Solvent: 2P:1A. Genotypes: the same as Fig. 6, but in the male sex. Two chromatograms for each genotype are shown. Each chromatogram from three decapitated flies. Length of run: 20 hours.

ever, at the present stage. What should be stressed again is the extraordinary constancy of results which makes it possible to maintain that each genotype has a characteristic biochemical pattern as revealed by this procedure.

Patterns of Single Gene Differences.—The results so far presented indicate that each of the tested genotypes imparts a remarkable specificity to the body constituents of the flies which can be separated by paper chromatog-

raphy. It was not known, however, whether such differences could be related to the particular gene mutations marking the used strains or whether they were due to the action of the specific allele involved plus that of the remaining genetic background or the background alone. Since the mutant strains had been kept in the laboratory for many years, and, besides, some of them had been originally found in unrelated strains, it could well be that the amount of genetic difference between the mutant strains was really much greater than the one revealed by the presence of a morphological mutant. To get some evidence on this point it has been possible to take advantage of some special strains kindly provided by Dr. Jack Schultz. These were (see D.I.S. 25[7]): p 1 (brother-sister inbred since 1947 from Oregon-R-S); p 4 (*f* Oregon-R-S, derived from crosses in 1947 to inbred stock from Oregon-R-S, inbred since then); p 7 (*gt* Oregon-R, similar origin).

As it can be seen in the accompanying photographs (Fig. 5) clear-cut differences are to be seen between the said strains. Since here one is probably dealing with a common genetic background in all the strains and with single gene differences, it seems safe to conclude that the biochemical specificity as revealed by this procedure falls under the control of single genetic loci.

Patterns of Recessive Gene Heterozygotes.—To this stage the analysis had been carried either on strains morphologically identical but differing in their geographic origin or on mutants easily distinguishable at the morphological level. The fact that genotypic differences could be revealed by paper chromatography irrespective of whether they would or would not appear in the phenotype, led to testing whether one could distinguish by this method two individuals phenotypically similar, but one being homozygous for the dominant allele and the other heterozygous for a recessive mutant at the same locus. With this idea in mind, the chromatograms of several recessive gene heterozygotes were studied and compared with the patterns of the homozygous parental strains. The same recessive mutant was crossed to more than one wild type strain. The following wild type and mutant strains were used for this experiment: Canton, Samarkand, Oregon, *bt*, ci^W, ey^2, *shv*, *spa*, *ss*, ss^a, *vg*. In every tested case it has been possible to show that the heterozygous individuals give a chromatographic pattern which differs from the one of its wild type homozygous parent as well as from that of the homozygous recessive. Figures 6, 7, 8, bring evidence to this point and show that this is true irrespective of the wild type strain used (e.g., *ss* was crossed with the three strains mentioned above). By this technique it became possible, therefore, to uncover genotypic differences otherwise hidden by dominance.

Maternal Effects.—While analyzing patterns of heterozygous individuals it became evident that in some cases some constant difference was to be found between heterozygotes for the same gene derived from reciprocal

crosses. This was the case for the following combinations: $+/shv$ - $shv/+$; $+/vg$ - $vg/+$; $+/ss$ - $ss/+$, using as wild type strain anyone of the three above mentioned; in the case of *hairy* the two reciprocal hydrids could be distinguished only when using Samarkand as parental wild type strain. In such combinations, while the heterozygous individuals could always be

FIGURE 8

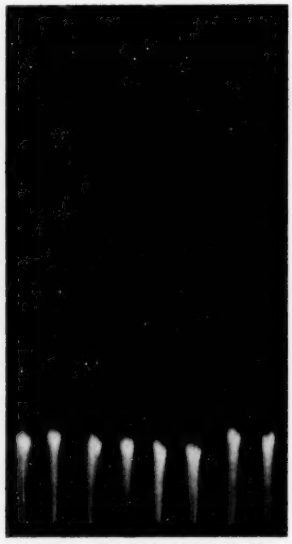

FIGURE 9

Fig. 8. Chromatographic patterns of fluorescing materials in *Drosophila melanogaster* males. Solvent: B:A. Genotypes: homozygous wild type Canton; *vg/vg;* F_1 from ♀ Canton × ♂ *vg;* F_1 from *vg* × ♂ Canton. Two chromatograms for each genotype are shown. Each chromatogram from three decapitated flies. Length of run: 20 hours.

Fig. 9. Chromatographic patterns of fluorescing materials in *Drosophila melanogaster* males. Solvent: 2P:1A. Genotypes: homozygous wild type Canton; *shv/shv;* F_1 from ♀ Canton × ♂ *shv;* F_1 from ♀ *shv* × ♂ Canton. Two chromatograms for each genotype are shown. Each chromatogram from three decapitated flies. Length of run: 20 hours.

distinguished from the homozygous dominant, the fluorescing pattern was more similar to that of the female parent than to that of the male. Figures 6, 7, 9, 10 show how such material effect can appear in the chromatograms. All the mentioned cases have been crosschecked using the two solvents and studying both sexes. No such maternal effect was found in the heterozygotes for the following mutants: *bt*, ci^W, *en*, *spa*, ss^a.

PLANT MATERIALS

In order to ascertain the range of applicability in other organisms of this technique for studying the biochemical specificity imparted by the genotype, similar investigations were carried on other animals and plants kindly made available to the writer. Some results obtained with plants will follow, as these give indications of a genotypic control of the biochemical specificity of various tissues of the same organism.

FIGURE 10

Fig. 10. Chromatographic patterns of fluorescing materials in *Drosophila melanogaster* females. Solvent: B:A. Genotypes: homozygous wild type Oregon-R; *ss/ss;* F_1 from ♀ Oregon-R × ♂ *ss;* F_1 from ♀ *ss* × ♂ Oregon-R. Two chromatograms for each genotype are shown. Each chromatogram from the decapitated females. Length of run: 20 hours.

Three tissues have been used: anthers, root tips, and leaves. Anthers were directly mashed onto the paper without any pretreatment using a flat glass pestle. One or more anthers were used so as to form a spot about $^3/_8$ in. in diameter; residual hard tissue was not removed. Root tips were thoroughly washed with tap water in order to remove completely any soil, inorganic and organic materials; they were then dried with filter paper, and cut to 1 cm. long pieces. From 4 to 8 such pieces, according to their thickness and juiciness, were directly mashed onto the paper with a glass pestle to form a spot of the mentioned size; hard parts were not removed. Leaves were thoroughly ground with a pestle in a mortar; with the aid of a capillary tube about 10 lambdas of the obtained juice were allowed to be absorbed by the paper. When leaves were too dry to allow this procedure some of the ground material was brought onto the paper with the aid of tweezers and enough of the oozing juice was allowed to get absorbed by the paper to form a spot about $^3/_8$ in. in diameter. The leaf debris was then removed. After the various mashed tissues and juice had been allowed to dry at room temperature, descending chromatograms were developed for 20 hr. at 25°C. using the said solvents. Most satisfactory separation was obtained from B:A.

Tomato (*Solanum lycopersicum L.*).—Chromatograms of anthers, leaves and root tips of a wild type strain, of the *male sterile*$_2$ mutant as described by Rick,[8] and of the heterozygote obtained by crossing the two homozygous strains were obtained. This recessive mutant was found in the Pearson variety of tomato. Its description is: "Smears of mature anthers reveal no pollen, either normal or aborted. Sections show concave anther walls and a small amount of degenerated material present in the locule. Meiosis proceeds normally. Microspores degenerate shortly

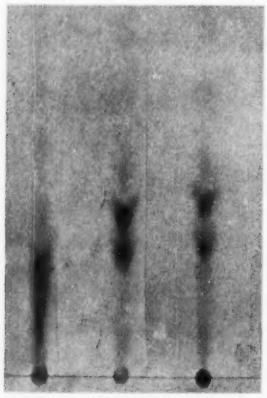

FIGURE 11

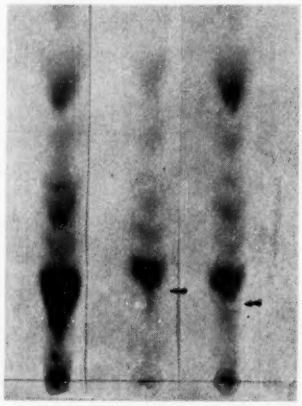

FIGURE 12

Fig. 11. Chromatographic patterns of ninhydrin-positive materials in muskmelon leaves. Solvent: B:A. Genotypes: homozygous wild type; *yg/yg;* heterozygous *+/yg*. Each chromatogram from a drop of leaf juice. Length of run: 20 hours.

Fig. 12. Chromatographic patterns of ninhydrin-positive materials in muskmelon root tips. Solvent: B:A. Genotypes: same as in Fig. 11. Each chromatogram from smeared root tips. Length of run: 20 hours. Arrows indicate whitish halo corresponding to very bright blue colored fluorescing substance present in *yg/yg* and the heterozygote.

after formation of the tetrads." The fluorescing, as well as the ninhydrin-positive patterns obtained from each of the three tissues were characteristic for each genotype, and the heterozygote could be easily identified in each case. Such identification was especially clear in the case of the root tips. Here the recessive mutant had two very clear fluorescing spots (R_f about 0.7 and 0.8) not present at all in the wild type strain; the heterozygous pattern, similar in every other respect to that of the dominant genotype, showed the presence of the two extra fluorescing spots present in the mutant. The ninhydrin positive material allowed, too, an easy recogni-

tion of the heterozygotes, as this gave a pattern intermediate between the ones of the parental strains.

Muskmelon (*Cucumis melo L.*).—In this material three strains have been studied: a wild type, a *yellow-green* mutant as described by Whitaker,[9] and the heterozygote for this recessive condition. The total chlorophyll content of normal green plants is slightly over twice that of the *yg* mutant. The ratio of chlorophyll *a* to chlorophyll *b* in normal green plants is 2.42, and in the yellow-green mutant 3.18. In the heterozygous condition, yellow green is completely recessive, in that there is no significant departure in the relationship of total chlorophyll content in the normal green plants and in the heterozygotes for the *yg* mutant. Chromatograms were obtained from leaves and root tips. Using both tissues distinctive patterns for fluorescing and ninhydrin-positive materials were obtained for the two parental strains; this made the recognition of the heterozygote possible. As can be seen in figures 11 and 12, while the heterozygous leaf gives a chromatogram practically indistinguishable from that of the homozygous recessive, in the case of the root tip chromatogram an intermediate pattern is observed. This is true for the fluorescing material also; the light-colored halo indicated with an arrow on figure 12 corresponds to a very bright bluish fluorescing area, which can be seen as very intense in the *yg* mutant, less intense but quite evident in the heterozygote, but completely absent in the normal strain.

DISCUSSION

Data here presented indicate that (1) the biochemical constitution of an organism or of its parts as revealed by the paper chromatographic technique is exceedingly constant and is largely independent of dietary and environmental conditions; (2) characteristic differences at the biochemical level can be found when different genotypes are compared; (3) the method is sensitive enough as to uncover genotypic differences masked by dominance phenomena; (4) single gene as well as multifactorial differences can influence in a definite way the biochemical makeup of an organism; (5) various tissues of an organism, give, as expected, different biochemical patterns; but when the patterns of the same tissue coming from organisms genetically different are compared, constant biochemical differences can be found irrespective of whether the morphology of the studied tissue is directly affected by the genotype involved; (6) maternal influence at the biochemical level and not affecting the morphological features can be uncovered with this technique.

No serious attempt has been made to recognize the chemical nature of the substances separating in the chromatograms, and this shall be the necessary prerequisite for more detailed work in the field. The only indirect piece of evidence on this problem is this: hydrolyzed Drosophila

and plant tissues have been analyzed with the same chromatographic technique, and after hydrolysis no difference was to be found, whether in the fluorescing or the ninhydrin-positive patterns, when comparing various genotypes. It is not known whether this is due to a masking effect of the great amount of aminoacids and other substances released by the hydrolysis and separating in the chromatograms. It appears, however, that part at least of this effect is due to the control imparted by the genotype to the building up of larger molecular structures, starting from simpler chemical substances common to every genotype. On this view the chromatography of untreated tissues would separate part at least of such large molecular structures and would thereby give a clue to and understanding of the biochemical specificity imparted by the genotype.

The idea that the genotype controls the specificity of proteins or of other large molecular structures is as old as the earliest speculations on the physiology of gene action. Some indirect evidence on this point has been brought by immunological studies and by grafting of tissues. Inbred strains or identical twins give identical reactions while this is not the case for sibs (see L. Loeb[10]). Taking advantage of the great sensitivity of the paper chromatographic technique, it seems feasible to carry this analysis a few steps further and perhaps to describe in known chemical terms products of a certain genetic condition. We do not know whether the substances isolated by this technique are to be considered excretion products, or cellular saps, metabolites or catabolites, or what definite constituent of the analyzed tissues. The remarkable constancy of results, on the one hand, and the fact that the tissue seems to fall under the biochemical control of the genotype, on the other hand, point to the particular significance of the described patterns. Results obtained in other organisms, such as fish, comparing the chromatographic patterns of a definite tissue (e.g. muscle) of different species (Buzzati-Traverso and Rechnitzer[11]), indicate, too, that the degree of biochemical similarity is somehow related to the degree of taxonomic relationship. This being the case, the precise chemical analysis of the observed differences could elucidate several obscure points concerning the physiology of gene action and phylogenetic affinities in chemical terms. Without indulging too much in speculations, however, it seems safe to maintain that this simple technique provides us with a very useful tool for genetic studies. The possibility of revealing genetic differences up to now not evident in the phenotype looks like the most promising result, with respect to theoretical as well as to practical applications. A series of other genetic problems, such as the comparison between mutants and their phenocopies, or the tracing back in the course of development of the biochemical differences evident in the adult stage, is now open to analysis from this angle.

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ALLELES AT A MUTABLE LOCUS FOUND IN POPULATIONS OF WILD HOUSE MICE (MUS MUSCULUS)

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Previous work[1] has suggested that a high rate of mutability of one locus or area of a chromosome, resulting in its ability to produce a variety of alleles, may be causally related to its degree or kind of activity in controlling key processes in development. The facts cited were that one locus, known as *T*, in a laboratory strain of mice, has mutated under observation to

produce a series of ten or more alleles of which eight have been analyzed genetically.[1] Several of these alleles which have also been studied embryologically[2] were found to play an important part in the determination mechanism which, in the early embryo, centers in the notochord-mesoderm material. The question thus arose whether the mutability was determined by factors peculiar to certain laboratory stocks in which this locus is maintained in a balanced lethal condition (T/t^n, T and t^n each being lethal and acting as alleles), or whether it was commonly a property of the locus. If the latter, then the locus should be mutable as well in wild populations of this species.

A plan was made for estimating the frequency of mutant sperm produced by males captured in the wild. This involves mating wild males with laboratory females which are heterozygous for a dominant at this locus. Such females, of genotype $T/+$, have short tails (a phenotype referred to as Brachyury) and when mated with males $+/t^n$ regularly produce compounds T/t^n which are tailless. t^n is defined as an allele at this locus which interacts with T to produce taillessness. Consequently the normal result of crossing wild male $+/+$ by $T/+$ females would be offspring $+/+$ (normal) and $T/+$ (Brachy), but should a t^n (mutant) sperm produced by the wild male effect fertilization then a T/t^n (tailless) offspring might be expected. The high rate of such mutations to t in domestic stocks would lead to the expectation that if the locus were equally mutable in the wild, then mutants might be found at a rate of 1 per 313 to 1 per 493 offspring observed at birth. One of these estimates is based on 11 exceptions in 3451 offspring observed at birth in a balanced lethal stock, the other on 7 of these which proved to be different t^n mutants.

Such an experiment was begun with ten males from a population which had originated from wild mice captured in suburbs of New York and Philadelphia and maintained without outcrossing[3] by Dr. Howard A. Schnieder, who kindly furnished us with stock. Preliminary results of these tests showed[4] that of the eight males from this first sample which were fertile, five were themselves heterozygous for a t^n allele and thus could not be used for the intended test. The remaining three produced 63 normal 52 Brachy and one tailless offspring. The tailless exception, however, proved to be not T/t^n but a phenotypic variant of genotype $T/+$. Consequently no information was obtained on the point at issue, namely frequency of t^n mutants in sperm of $+/+$ males. In the meantime the first animal tested from a second wild population (from Mystic, Conn.) also proved to be $+/t^n$. This discovery of an unexpectedly high frequency of $+/t^n$ heterozygotes among wild mice caused a change in the plan of the experiment, the purpose of which now became to measure the frequency of $+/t^n$ heterozygotes in wild populations to determine whether or not the t^n alleles found in the wild were of independent origin and thus to obtain an estimate of the numbers of *different* t-alleles in the wild.

Although observations planned for the above purposes would not of themselves reveal the rate at which mutations had occurred from + to t^n, they should yield information on the variety of forms of t^n occurring in different natural populations and thus permit comparison of the potentialities of the locus in domestic and wild populations. The new experiments included also tests of lethality or viability of each t^n allele found, of segregation ratios $T:t^n$ and $+:t^n$ which we had found to be abnormal in males of laboratory strains,[6] and of sterility effects of different t alleles in male compounds such as t^x/t^n. These effects of the t^n alleles would affect the likelihood of persistence of such alleles in wild populations and, together with information on the relative fitness of $+/t^n$ as compared with $+/+$, would constitute essential data for computing the adaptive value of different t^n genotypes and thus could yield indirect evidence of mutability of this locus.

TABLE 1

SOURCES OF THE SAMPLES OF WILD POPULATIONS AND THE CLASSIFICATION OF WILD MICE TESTED FOR ALLELES AT LOCUS *T*

SAMPLE NO.		GENOTYPES FOUND +/tw	+/+
New York 1 (confined population Rockefeller Institute)	12 ♂♂	7	5
New York 2 (70 W. 106th St.)	1 ♂	1	..
Connecticut 1 (Mystic)	3 ♀♀	1	2
Vermont (Norwich)	4 ♂♂	..	4
Wisconsin 3 (Madison)	6 ♂♂	1	5
Wisconsin 1[a]	4 ♂♂	..	4
Wisconsin 2[a] (Madison)	10 ♂♂	..	10

[a] Samples contained animals with minus modifiers of Brachy simulating effects of *t*-alleles and other genes indicating contact with laboratory populations, hence not suitable for detecting heterogeneity at locus *T*.

Experimental Methods.—To accomplish these purposes samples of wild mice were obtained from the sources shown in table 1 and mated with $T/+$ animals from our colony. At first the chief source of $T/+$ was our line BT, Brachy in the 11th–13th brother-sister generation. Later it was found that better fertility was obtained when the $T/+$ animals were the offspring of a cross between the BT Brachy line and another equally inbred Brachy line known as Line 11. Breeding of wild mice in captivity was facilitated by the use of exercise wheels of the kind described by Schneider.[3] Otherwise wild mice were housed and fed like the laboratory stocks and except for wildness reacted to conditions like the domesticated mice.

When from such test crosses tailless progeny appeared, these were tested to determine whether they contained a t^n allele. If such animals are T/t^n, then when crossed with tailless animals of the known balanced lethal lines T/t^0 or T/t^1 only tailless (T/t^n and T/t^0 or t^1) and normal-tailed (t^n/t^0 or t^1) progeny appear, never any Brachy. If tailless offspring from a wild parent are not T/t^n but, as several proved to be, $T/+$, then Brachy ($T/+$) progeny also appear when they are test-crossed to T/t^0 or T/t^1. When tailless offspring from a wild parent have been shown to be T/t^n, they are then mated *inter se*, both to maintain the new mutant allele and to determine whether it is lethal or viable when homozygous. Segregation ratios from T/t^n males by $T/+$ and $+/+$ females are obtained from appropriate crosses; and males t^w/t^0 and t^w/t^1 are tested for fertility by mating with normal females of proved fertility.

Results.—Observations of all the above kinds have been obtained from one wild population, and a part of them from samples from four other wild populations. The evidence indicates that four of the five populations tested contained at least one t^n allele.

Population Samples from New York.—The first sample from this population, furnished by Dr. Schneider, has already been referred to. After the first sample had been analyzed, we obtained a second sample from the same population. Tests of twelve males and of the descendants of some of them are shown in table 2. A sample of females was also tested and $+/t^w$ heterozygotes found among them, but due to smaller numbers of offspring obtainable from individual females, the frequency of $+/+$ among them has not been established, hence the comparative frequency of $+/t^w$ and $+/+$ is available for males only.

The seven males diagnosed as $+/t^w$ are clearly distinguished from the five diagnosed as $+/+$. Each of the seven gave tailless offspring in a frequency exceeding, usually by wide margins, the frequency of Brachy offspring. Adequate tests of these F_1 tailless offspring have been carried out for the first four males. All of the F_1 tailless tested have been diagnosed as T/t^w since when crossed with tailless from known balanced lethal lines (T/t^0 and T/t^1) they have never yielded a Brachy offspring which would be expected if F_1 tailless were of genotype other than T/t^w. Each F_1 tailless male thus proved to be T/t^n was then crossed with an F_1 tailless female, similarly tested, and derived from the same wild male. The F_2 offspring should reveal whether the t^w allele in each case is lethal or viable. If lethal, the cross $T/t^w \times T/t^w$ should yield only tailless offspring, since TT and t^wt^w would die before birth. If viable, the latter genotype should survive as non-tailless. The F_2 populations from three wild males gave clear results indicating that these t^w alleles were viable and give normal (or nearly normal) tails when homozygous. The F_2 population from one male has given 67 tailless with two animals dead at birth, one of which ap-

TABLE 2

RESULTS OF TESTING MALES OF WILD POPULATION NEW YORK 1

WILD PARENT	PROGENY BY BRACHY $T/+$			PROGENY FROM F_1 TAILLESS T/t^w TESTED BY T/t^0 AND T/t^1			PROGENY FROM F_1 TAILLESS × F_1 TAILLESS		
	NORMAL	BRACHY	TAILLESS	NORMAL	BRACHY	TAILLESS	NORMAL	BRACHY	TAILLESS
♂4922 $+/t^{w1}$	23	2	20	42	..	50	1[a]	1[a]	67
4927 $+/t^w$	29	5	20	21	..	54	10		14
4928 $+/t^{w2}$	83	3	57	23	..	93	51		75
4929 $+/t^w$	14	2	7	43	..	64	6		9
4930 $+/t^w$	6	..	4	...					
6380 $+/t^w$	47	4	38	...	..	...	...		..
6385 $+/t^w$	5	1	2	...	..	...	...		..
Totals from 7 ♂♂ $+/t^w$	207	17	148	129		261	67[b]		98[b]
4923 $+/+$	23	28	...	...	..	...			
4925 $+/+$	14	14	1[c]	4	9!	10			
4926 $+/+$	26	11	...						
6386 $+/+$	21	11	...						
6387 $+/+$	23	21	...						
Totals from 5 ♂♂ $+/+$	107	85	1						

[a] Dead at birth. [b] Totals from viable alleles only. [c] Tested = $T/+$.

peared to have a normal tail, the other a Brachy tail, although tail length diagnosis is not wholly accurate on dead and stillborn mice. The tentative interpretation is that this t^w allele is lethal but that occasional homozygotes survive until near the time of birth. This allele is obviously different from those which are viable when homozygous and has been designated as t^{w1}. One viable allele, that from wild male 4928, has been designated t^{w2} and retained for further study. The viable alleles from 4927 and 4929 may be the same as 4928 or may be different; there is no simple way of distinguishing at present. The chief conclusion from the F_2 tests is that the wild sample contained at least two different t^w.alleles, one lethal and one viable. Whether these originated in the two different wild populations, one from New York and one from Philadelphia, that contributed to the confined population in Dr. Schneider's colony, or whether one or both originated in captivity cannot now be ascertained.

One of the five males in table 2 diagnosed as $+/+$ gave one exceptional tailless offspring. This animal was tested by T/t^0 and T/t^1 and gave Brachy offspring proving it to be $T/+$, hence an overlap. Tail length of other F_1 Brachy offspring from this cross appeared to be similar to that found in the inbred Brachy test stock so the one exception may have had a mutant modifier of Brachy. Two wild males gave a considerable excess of normal progeny, suggesting that some of these might be $T/+$, i.e., overlaps with plus modifiers of Brachy. Progeny tests of seven such F_1 normals by normal mates gave 213 normal tailled offspring, hence no evidence that any F_1 normal was $T/+$.

Behavior of t^w Alleles from the Wild.—The new t^w alleles obtained from this population resemble in their properties tested so far the t-alleles from laboratory populations.[1] Wild males $+/t^w$ produce a great excess of t^w sperm. The ratio of t^w to $+$ sperm is given approximately by the ratio of tailless T/t^w to Brachy $T/+$ offspring when $+/t^w$ male is mated to $T/+$ female. For all $+/t^w$ wild males tested this was 148:17 (table 2) a ratio of about 8 t^w to 1 $+$. Similar behavior has been shown by t^0, t^1, t^2, t^9, and t^{12}, all lethals, among the laboratory alleles. In the wild alleles, the viables give just as extreme ratios as the lethal one.

Alleles from the wild in compound with alleles from laboratory populations t^0/t^{w1}, t^1/t^{w1}, t^0/t^{w2}, t^1/t^{w2} give normal-tailed individuals in both sexes but the males of all combinations tested are mating sterile. Twenty-five such males mated individually for four months with an average of 3–4 females of proved fertility have not produced a single offspring or pregnancy, although they have been observed to copulate and to form copulation plugs. This type of mating sterility has previously been found to accompany two different lethal alleles in compound (t^0/t^1).[6]

Occasionally a lethal compounded with a viable (t^0/t^3 or t^1/t^3) yields quasi-sterile males.[7] The tests applied to date do not exclude the possi-

bility that occasional sperm of t^0/t^w or t^1/t^w males may be functional as revealed by examination of cleaved eggs in special tests.[8]

In summary, then, a sample of one wild population bred for several generations in captivity was found to contain at least two different alleles at locus *T* which behave like other alleles which had arisen by mutation in laboratory stocks. The mutant alleles, in male heterozygotes, are transmitted in much higher frequency than the normal alleles. This is probably responsible, in part at least, for the high frequency of heterozygotes, 7 out of 12 males tested, in the sample.

A single male, caught wild[9] in an apartment at 70 West 106th Street, Manhattan, is the sole representative tested from a second New York population. This animal was paired with several Brachy females and escaped after five days, but fortunately had fertilized one female who bore a litter of 5 normal, 1 Brachy, and 2 tailless. One normal F_1 male tested by Brachy has given 8 normal and 12 tailless from which a T/t^{w5} stock is being bred.

Population Samples from Wisconsin.—Three samples of wild males from Wisconsin were sent to us through the kindness of Dr. John Emlen and Mr. Charles Southwick of the Department of Zoology, University of Wisconsin. Sample 1 was from an enclosed population maintained by Dr. Emlen; sample 2 was trapped in the feed room of the Zoology Department; and sample 3 contained animals from three sources: Nos. 6083, 6084, 6085 were trapped in the genetics barn, Madison; Nos. 6086 and 6087 were trapped in a chicken house (U.W.Z.) at the University, and No. 6089 was from a confined population. These samples can probably not be taken as representative of wild populations because of the proximity of the source to laboratory mice. Two animals in sample 2 proved to be heterozygous for albinism (*Cc*) and one in sample 3 (6083) was heterozygous for brown (*Bb*), mutants not known to occur in the wild. However, the results of testing these samples revealed one of the difficulties attending the identification of *t*-alleles in untested populations which will be important in future work and are thus reported here.

Sample 1 when tested yielded the results expected if all of the wild parents were +/+. However each of two wild males produced one tailless offspring which might be $+/t^n$ due to a mutant sperm, or might be a phenotypic variant of $T/+$, or a mutant at another locus. It was noticed that the Brachy offspring from each of the wild males in this sample had shorter tails than in the test stock used in which the average tail length was from $^1/_2$ to $^3/_4$ of normal length, indicating that the tailless animals probably represented the extreme in phenotypic modification of the $T/+$ genotype. Direct test of the surviving tailless animal by T/t^0 and T/t^1 has not yet yielded a decisive answer to this question. This experience indicated that classification of tail length of all newborn Brachys in such tests would provide useful information, and this was thereafter done routinely

TABLE 3

RESULTS OF TESTING MEMBERS OF SAMPLES 1 AND 2 OF WILD POPULATIONS FROM MADISON, WISC.

WILD PARENT	PROGENY BY BRACHY			TAIL-LENGTHS OF F_1 BRACHYS					PROGENY FROM F_1 TAILLESS TESTED BY T/t^9 AND T/t^{11}		
	NORMAL	BRACHY	TAILLESS	1/8	1/4	1/2	3/4	7/8	NORMAL	BRACHY	TAILLESS
Sample 1											
♂5786	14	8	...	1	2	4	1				
♂5787	14	14	1[a]	2	4	8	..				
♂5788	17	9	1	1	..	8	..				
♂5789	12	22	...	..	4	16	2				
Totals Wisc. 1	57	53	2	4	10	36	3				
Sample 2											
♂5845	6	12	1[b]	..	2	6	4	..	..	1	3
♂5847	37	36	...	..	..	16	19	1	..	...	..
♂5848	20	22	9[c]	1	7	14	..	..	5	13!	12
♂5849	10	4	...	..	..	1	3	..	..	..	..
♂5850	30	22	2	..	1	16	5	..	..	..	..
♂5851	11	19	2[d]	1	2	14	2	..	2	7!	4
♂5852	8	8	1[e]	..	1	2	5	..	..	..	..
♂5853	17	16	1[b]	..	..	14	2	..	2	2!	2
♂5854	30	12	14[f]	3	5	4	..	..	17	11!	26
♂5855	30	33	1	..	2	4	24	3	..	..	..
Totals Wisc. 2	199	184	31	5	20	91	64	4			

[a] Dead at birth. [b] Tested = $T/+$. [c] 3 ♀ 1 ♂ tested = $T/+$. [d] 1 tested = $T/+$. [e] Died; 2 F_1 normals tested = $+/+$. [f] 5 tested = $T/+$.

on a rather rough scale as shown in table 3. The results leave no doubt that the males of sample 1 contained modifiers which shorten the tail-length of $T/+$, occasionally resulting in taillessness. This effect is even more pronounced in the offspring of males of sample 2 which showed continuous gradation from tailless to almost normal. Here direct tests of 12 tailless by T/t^0 and T/t^1 yielded Brachy offspring in each case, indicating that these F_1 tailless were $T/+$. Again average tail length of the F_1 Brachys was lower than in the test Brachy stock indicating presence of minus modifiers of Brachy in the wild parents. Although some of the untested F_1 tailless may have contained t^n mutants, identification of these among the many tailless overlaps of the $T/+$ would be difficult and always attended by uncertainty, since some of the tailless are born dead or prove to be sterile. The best judgment of samples 1 and 2, therefore, is that (1) no t^n mutants were identified; (2) such samples which contain minus modi-

TABLE 4

RESULTS OF TESTING MEMBERS OF SAMPLE 3 OF WILD POPULATIONS FROM MADISON, WISC.

	PROGENY BY BRACHY			TAIL LENGTHS OF BRACHYS				
WILD MALE	NORMAL	BRACHY	TAILLESS	1/8	1/4	1/2	3/4	7/8
6083	11	25	..	...	1	9	8	7
6084	17	8	..	2	...	6	..	..
6085	24	11	..	...	...	2	7	2
6086	13	4	..	...	...	1	2	1
6087	27	22	..	...	...	6	12	4
Total +/+	92	70	..	2	1	24	29	14
6089 $+/t^{w4}$	16	1	10					

fiers of Brachy are unsuitable for the detection of $+/t^n$ heterozygotes or of t^n mutants among the gametes of the wild parent. Both of these samples are thus to be excluded from the wild populations tested. It is to be noted that both depart from the expected behavior of wild populations by having modifiers which increase rather than decrease the effect of the Brachy mutation.

The males of sample 3 were of three types. Four of them tested as though $+/+$ and gave no evidence of having minus modifiers of tail length. One (6084) produced two very short-tailed offspring and the others were shorter than average; he is of the indeterminate type as in sample 2. One male (6089) gave the typical result expected of $+/t^w$, that is, many tailless and few Brachy offspring. Four F_1 tailless males tested by T/t^0 (Line A) have given 43 tailless only, indicating that the new allele (t^{w4}) may be the same as t^0 or that the compound t^0/t^{w4} is lethal. Three F_1 normal females tested by Brachy proved to be $+/t^{w4}$. Male 6089 was from a confined

population originating in the wild, hence the allele found in him was probably from a wild population and is retained in stock as t^{w4}. This allele is probably lethal and may be a recurrence of t^0.

The outcome of the tests of the Wisconsin samples is thus one t^w allele from one wild population.

Tests of Wild Mice from Connecticut.—We are indebted to Mr. Walter C. Morgan, Sr., for three females trapped in the feedroom of his poultry house at Mystic, Conn. When tested by a Brachy male two of these gave only normal and Brachy offspring (9 normal and 15 Brachy with tails ranging from $^1/_2$ to $^7/_8$ of normal length), while one gave 10 normal, 4 Brachy ($^5/_8$–$^7/_8$ of normal length), and 3 tailless. Three of these F_1 tailless offspring, both males, have been tested sufficiently to diagnose them as T/t^{w3} and the wild parent as $+/t^{w3}$. By T/t^0 and T/t^1 tailless females these males have given 35 tailless and 31 with normal or nearly normal tails. Some of the latter were blunt but none was clearly Brachy. These males were each crossed to stock Brachy females and to date have produced 49 normal, 1 Brachy, and 67 tailless. This indicates that each F_1 male is T/t^{w3} and produces, as in the case of other *t*-alleles, a great excess of t^{w3} sperm, resulting in a great excess of tailless over Brachy offspring from the test cross. Two of these males tested by a standard normal tailled stock (Bagg albino) have given 58 normal only, that is, 58 t^{w3} gametes tested. Matings of $T/t^{w3} \times T/t^{w3}$ have so far yielded 61 tailless offspring and one exceptional normal tailled one.

Wild Mice from Vermont.—Through the cooperation of Dr. M. T. M. Rizki of Dartmouth College four wild males trapped near Norwich, Vermont, have been tested as follows:

TABLE 5

RESULTS OF TESTING MEMBERS OF VERMONT POPULATION

WILD MALE PARENT	PROGENY BY BRACHY			TAIL LENGTH OF F_1 BRACHYS				
	NORMAL	BRACHY	TAILLESS	$^1/_8$	$^1/_4$	$^1/_2$	$^3/_4$	$^7/_8$
6615	6	2	...	...	...	...	2	...
6614	5	4	...	...	...	...	4	...
6616	31	29	...	...	...	1	25	3
6825	4	6	...	...	...	1	5	...
Totals	46	41						

No tailless progeny appeared and the tail length of the F_1 Brachys indicates that neutral or plus modifiers are present, a favorable situation for detecting *t*-alleles.

Wild Mice from Maine.—Mr. Darien Terrile of Bates College has caught and tested five wild mice from the neighborhood of Lewiston, Me. He reports that the offspring are 25 with normal tail and 15 Brachy. The small samples from Maine and Vermont are the only ones from favorable wild populations which have not yielded at least one t^w heterozygote.

Other Variations in Wild Mice.—In addition to heterogeneity at locus *T*, the samples from wild populations examined superficially were found to contain the following variations:

1. *Belly color*—Sample 1, New York, contained animals with three different belly colors: white, yellow, and gray. The latter two colors appeared also in each of the three Wisconsin samples. The few animals observed in the other samples (New York 2, Connecticut, Vermont) had gray bellies. The belly color differences found are like those associated with the alleles A^{L} and A in domestic populations. White and yellow belly characters of the wild mice behaved as dominant to A and to a although no detailed study was made. It has commonly been assumed that in the U. S. gray belly is the wild type. This is obviously not uniformly the case.

2. *White spotting*—Sample 1, New York, contained some animals with a white blaze on the forehead, others had a small white spot in the center of the belly; white tail tips were noted on several. White spotting on the tail was noted in several of the Wisconsin specimens. No attempt was made to study the inheritance of these variations.

The impression was gained from observation of several samples of wild mice that wild populations may be polymorphic in several ways.

Discussion.—The original object of this investigation, namely to get an estimate of the frequency of mutation at one locus in wild mice, has not been achieved, and it is obvious that since some populations contain large numbers of animals already heterozygous for the locus to be tested, while others contain modifiers which simulate the effect of an allele at this locus, the accumulation of the necessary data will probably be slow. Nevertheless a new result of interest was obtained, namely that four out of five wild populations which were suitable for study contained an allele of the t^{w} type which behaved like the alleles which had arisen by mutation in the domestic stocks. It may be assumed therefore that many wild populations are heterogeneous for alleles at this locus. The apparent commonness of such alleles in the wild may be due to any one of several different factors, or to a combination of certain of them: (1) to a high mutation rate of + to t^{w} for which we have evidence from balanced lethal stocks but no evidence from nature; (2) to a high transmission ratio of t^{w} as compared to +, which certainly occurs in males $+/t^{w}$ and could account for the maintenance of t^{w} alleles above equilibrium even with a low mutation rate; (3) to selective advantage of $+/t^{w}$ heterozygotes for which we have no information at present. Experiments to test this last hypothesis are in progress.

It is obvious that the method used here for revealing heterogeneity at a well-known locus and for distinguishing among lethal alleles found in the wild may be found useful in characterizing and in estimating the dimen-

sions of individual wild populations. Our observations also raise the question as to the degree to which wild populations of this commensal are isolated from each other and from domestic or laboratory populations and suggest some of the means for studying such questions. The sterility of males carrying different *t*-alleles, e.g., t^w/t^x, and the excess of sperm carrying t^w as compared to + which have now been shown to apply to alleles from the wild and consequently to be characteristic of this locus rather than of particular laboratory populations, must be related to the means by which the alleles are maintained in nature and perhaps to particular isolating mechanisms. Finally, the questions raised at the beginning of this paper concerning the relation between the ability of this locus to produce a variety of alleles and its function in development are now posed in a wider context, since these properties appear generally connected in wild populations.

Summary.—An attempt to determine whether a locus, *T*, known to be mutable in balanced lethal stocks of laboratory mice, was also mutable in populations of wild mice led to the testing of samples from seven such populations. In each case wild mice were tested by the mutant form Brachy, $T/+$; and tailless animals of genotype T/t^w were looked for among the offspring, t^w being a mutant allele from the wild. Five samples were found suitable for yielding good data. Four of these contained animals heterozygous for such alleles, $+/t^w$. Two such alleles, t^{w1} (lethal) and t^{w2} (viable) were identified in one sample of composite origin. One allele each was found in the other three samples. Only one sample of four animals from Norwich, Vt., did not contain a t^w allele; another small sample (5 animals) tested at Bates College, Lewiston, Me., likewise gave no *t*-allele.

It was concluded that many wild populations are heterogeneous for alleles at the locus tested. Whether this is due to high mutability of this locus, to the high ratio in which t^w alleles were shown to be transmitted by males $+/t^w$, or to selective advantage of $+/t^w$ heterozygotes are questions which remain to be studied.

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EFFECTS OF ALKALI METAL IONS ON DEVELOPMENT OF DROSOPHILA, WITH SPECIAL REFERENCE TO LITHIUM-INDUCED ABNORMALITIES*

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Introduction.—Attempts were made to increase the lithium concentration of the tissue of *Drosophila melanogaster* prior to irradiation with thermal neutrons.[8] In the process of these experiments it was noticed that specific abnormalities were occurring in adults fed during larval stages on lithium-containing medium. A survey of the literature showed that Mann[11] in 1923 had reported that concentrations of 0.004 to 0.007 *M* Li_2CO_3 retarded larval and pupal development of *Drosophila melanogaster*. Aside from this paper no changes in developmental behavior following Li-treatment have been reported for *Drosophila*.

Material and Methods: Tolerance of Drosophila to Salts of Some Alkali Metals.—*Drosophila melanogaster* were raised on a culture medium made according to the following formula: 1 g. $MgSO_4 \cdot 7H_2O$; 6.2 g. propionic acid; 15 g. type 2019 Fleischman's brewers' yeast; 23 g. Difco Bacto-Agar; 110 g. Quaker's enriched degerminated yellow cornmeal; 170 g. Grandma's molasses; 1250 g. water. To this medium varying amounts of LiCl, NaCl, KCl, $NaH_2PO_4 \cdot H_2O$ or KH_2PO_4 were added for different experimental series. The cultures were $^1/_2$ pint bottles containing 50 cc. of food inoculated with a non-pathogenic strain of yeast, *Candida albicans*. These were started with two males and a female of a highly inbred Canton Special wild type stock of *Drosophila melanogaster*. Cultures were kept at 21°C. and the P_1 individuals removed after 8 days. Twenty days after the initial cross the number of F_1 individuals was determined and compared with the number of flies in control cultures.

Results.—The results are shown in the graph of figure 1. From examination of this graph one can conclude that lithium is far more toxic than any of the other salts tested. Under the experimental conditions described, no F_1 flies appear in cultures containing LiCl concentrations higher than 0.022 *M*. Even at lower concentrations the F_1 yield per culture is always below the control yield. On the other hand toxic concentrations of NaCl are not reached until molarities twenty times higher than those of lithium. This indicates that the effect of the lithium ion is specific rather than one due secondarily to osmotic or hydration phenomena. The lower arm of the curve for KCl is steeper than that of NaCl, and it appears that K^+ is more toxic than Na^+. The points representing the $NaH_2PO_4 \cdot H_2O$ and KH_2PO_4 series appear to follow the same relationship and consequently a single

curve is drawn. It is concluded here that the toxicity of the salts is due to the anion since chlorides of both salts are less toxic. The sex ratio for all the experimental series appears normal except in the case of the two alkali metal phosphates where the percentage of males is depressed at concentrations above 0.08 *M*.

Developmental Abnormalities Following Li-Treatment.—The sex ratio and appearance of F_1 individuals from medium containing 0.01 *M* LiCl are normal. However, *Drosophila* reared upon medium containing 0.02 *M*

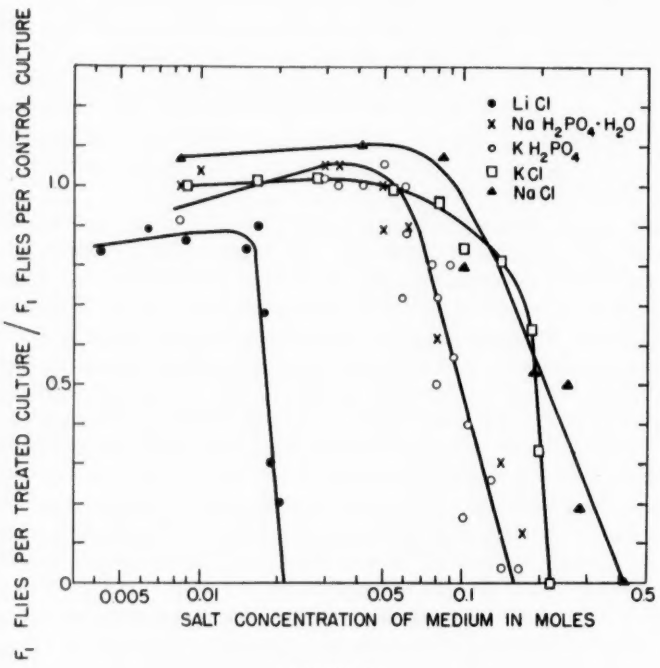

FIGURE 1

LiCl show a great prolongation of larval and pupal development of the F_1 individuals and for this reason the majority were not scored in the previous experiment. Normally at 21°C. development from egg to adult takes about 10 days. F_1 individuals under the above conditions took a minimum of seventeen days to reach the adult stage. In some cases the developmental time was three times the control value. The sex ratio of the late hatching adults from 0.02 *M* LiCl cultures was markedly disturbed (141 males to 5 females). Sixty nine per cent of the males had abnormal geni-

talia. The genitalia were either missing or were rotated. In the majority of cases rotation was 180°, although cases of 90° rotation were abundant. In one specimen the genitalia were duplicated yielding two sets of genital arches and associated anal plates, claspers, and penis. Dissections showed that the terminalia of genitalialess males were within the abdomen and in some cases were found floating freely in the haemolymph as far forward as sternite three. The terminalia were represented by a sphere attached by the posterior ejaculatory duct to the rest of the reproductive system. The ejaculatory bulb, anterior ejaculatory duct and accessory glands looked normal, but the testes and seminal vesicles were very pale-yellow with darker yellow blotches and were knotty in outline. The sphere contained internally numerous bristles, the claspers, and penis, all pointing toward the lumen.

The wings also showed characteristic modifications. The anterior cross-vein was missing, the junction between longitudinal veins 2 and 3 was disturbed, and longitudinal vein 5 was interrupted before reaching the wing edge. Notches often occurred at the wing tips and lateral edges. Less characteristic abnormalities include: almond-shaped eyes, missing scutellar bristles, mottling of the black pigmentation of the tergites, swollen metatarsi, and twisted femurs.

The few normal-looking males were fertile and produced normal appearing progeny. The five females were dissected and were found to have rudimentary ovaries, but appeared to be otherwise normal.

Discussion and Conclusions.—Morphogenetic abnormalities following Li-treatment were first described for sea urchin embryos by Herbst.[7] The manifold abnormalities in the embryological development of various organisms brought about by lithium have been admirably reviewed by Needham[12] and Brachet.[3] Lithium is known to inhibit respiration, and it poisons certain glycolytic reactions occurring in the developing embryo.[9] MacLeod, *et al.*,[10] have demonstrated that lithium ion inhibits both aerobic and anaerobic pathways of the degradation of glucose to lactic acid in mammalian spermatozoa, but stimulates aerobic glycolysis in testicular tissue. Among the alkali metals lithium has the smallest ionic radius and the highest degree of hydration. Its ability to flocculate colloids is well known. Raven[14] has pointed out the densifying action of lithium on cytoplasm. This increase in density is no dehydration phenomenon, but is thought to be due to a change in the submicroscopic structure of the cytoplasm. The increased density of cytoplasm may interfere with the displacements of cytoplasm normally occurring in the developing embryo.

The abnormal condition of the male terminalia resembles in certain respects the phenotype produced by the mutants *abdomen rotatum*,[1] *rotated-penis*[4] and *twisted*.[5] Rapoport[13] has produced dextral and sinistral rotation of the abdomen of *Drosophila melanogaster* following treatment with

para hydroxy and amino substituted benzenes. Lithium-induced abnormalities of the terminalia probably owe their origin to developmental failures occurring at some stage during the characteristic 360° clockwise rotation of the extreme posterior part of the male pupa of *Drosophila melanogaster*.[6] Lithium must produce its effect on the terminalia at a stage of development which in normal males occurs sometime prior to or at 30 hours after puparium formation, for the testes attach themselves to the seminal vesicle primordia at this time[2] and rotation occurs concurrently. The reason for the striking sexual dimorphism in lithium sensitivity cannot be explained at the present time.

Summary.—The tolerance of *Drosophila melanogaster* to salts of some alkali metals was studied. It was concluded that the lithium ion is by far the most toxic, while K^+ is slightly more toxic than Na^+ at high concentrations. The time required for the completion of development for *Drosophila* fed on a medium containing 0.02 M LiCl is increased two to threefold. F_1 adult populations consist mainly of males. Characteristic abnormalities are produced involving the male terminalia and wing venation.

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*SEROLOGICAL DIFFERENTIATION OF THE HOMOZYGOTES AND HETEROZYGOTES IN BACK-CROSS BIRDS FOLLOWING A SPECIES CROSS IN COLUMBIDAE**

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The existence of species-specific antigens on the erythrocytes of *Columba guinea*, the triangular spotted pigeon, in contrast to *C. livia*, the common domestic pigeon, and their segregation in the progeny of matings of the species hybrids and selected back-cross hybrids to livia have been previously reported.[1] Five different antigenic substances specific to guinea have been identified as presumed hereditary units, and are called A, B, C, E, and F.[1] Back-crosses to guinea to isolate the species-specific substances of livia in contrast to guinea have not been possible under the conditions existing in this laboratory. However, an indirect method for identifying five of the specific substances of livia, respectively antithetical to the five specific substances of guinea, and the resulting ability to detect the different heterozygotes are described in this paper.

Materials and Methods.—The species-specific cellular antigens A, B, C, E, and F of the red blood cells of guinea, in contrast to livia, have been maintained in back-cross hybrids by back-crosses to livia and by mating the back-cross hybrids *inter se*. As reported elsewhere[2] two birds, G284X and G307H, from parents both possessing antigen C were found to be undoubtedly homozygous for C (*CC*), since in matings to livia these two birds produced 23 and 15 offspring, respectively, all possessing antigen C. When mated together these two birds, therefore, produced offspring homozygous for antigen C and these offspring were those used as the homozygous CC birds in these tests.

The immunological procedures were similar to those described elsewhere.[2, 3] Nine antisera used in these tests for specific livia substances were produced in rabbits against the red blood cells of livia, but most of the studies were made with one anti-livia serum, 150S4. The reagents for the contrasting antigens of livia were prepared by absorbing the anti-livia sera

with the cells of back-cross hybrids homozygous for the gene or genes producing one of the species-specific antigens of guinea, as CC. For the absorption, the antisera were diluted 1:60 with saline, and mixed with appropriate cells, as CC, in the ratio of 4 volumes of packed cells to 1 volume of antiserum. The absorbing cells were divided into 4 equal parts and the absorptions were made serially for 5 minutes, 90 minutes, 90 minutes, and overnight, respectively. The first three absorptions were conducted at room temperature and the last at refrigerator temperature, about 4°C.

The possession of antigen C, or another antigen of guinea, by the back-cross hybrids was determined by agglutination of the cells with the specific reagents prepared as described elsewhere.[2, 3] For example, anti-guinea serum absorbed with the cells of back-cross hybrids possessing, respectively, the antigens of guinea, A, B, E, and F, was presumably specific for antigen C. In addition, antisera to the cells of back-cross hybrids possessing only C, when absorbed with corpuscles of livia, were also specific for cells possessing antigen C. Thus, reagents prepared by two different methods were available for demonstrating the cellular antigen C in guinea and the back-cross hybrids. Reagents for the other species-specific antigens of guinea were prepared by parallel methods.

Results.—If livia possesses a gene (or genes) allelic to a gene (or genes) in guinea which effects the C substance, and the gene or genes of livia produce an antigenic substance on the red blood cells in contrast to the C substance of guinea, then theoretically, back-cross birds which are homozygous for antigen C should differ from livia not only in the possession of the C substance of guinea but they should lack the contrasting antigenic substance to C postulated for livia. If this is the case, it should be possible to demonstrate this contrasting substance of livia with reagents prepared by absorbing anti-livia serum with pooled cells of the homozygous *CC* birds. These cells of the homozygotes presumably possess all the antigens of livia except those contrasting to C of guinea; all antibodies of an antiserum to livia cells should be removed in absorption by cells homozygous for *CC* except those antibodies specific for the substance of livia antithetical to C of guinea. This reagent should be specific for the contrasting antigen of livia and, therefore, should react only with the cells of the heterozygotes and of livia.

The reagent produced by absorbing each of the nine antisera to livia, when absorbed with the cells of birds homozygous for C (*CC*), strongly agglutinated the cells of livia and those of known heterozygotes. (All progeny possessing C from back-crosses to livia of birds possessing antigen C, of course, will be heterozygous for C.) The reactions of the four different kinds of cells (guinea, livia, *CC* and the heterozygotes) with the reagents prepared from anti-livia serum, 150S4, are given in table 1. The reagent prepared by absorptions with the cells of homozygotes for C failed to ag-

glutinate the cells of some birds resulting from the mating *inter se* of backcross hybrids possessing C, but was strongly reactive with the cells of all other offspring from these matings. Presumably, those cells which did not react with this reagent were from birds homozygous for C (*CC*), and, as expected, reagents produced by their use in absorptions of anti-livia serum paralleled the reactions of those made from cells of the known homozygotes for C. Further, four birds predicted to be homozygous by the serological test were mated to livia, and each of a total of 26 offspring (6, 6, 7, and 7 in the respective progenies) possessed antigen C as expected. From these results it is concluded that the red blood cells of livia possess an antigen antithetical to the C of guinea. This contrasting antigen of livia is called C′.

As may be seen in table 1, when anti-livia serum was tested with the cells of guinea (presumably *CC*), homozygous *CC*, heterozygous *CC′* and livia (presumably *C′C′*), agglutination occurred in a serum dilution of 1:7680

TABLE 1

SEROLOGICAL TEST FOR THE PRESENCE OF AN ANTIGEN OF *Columba livia* CONTRASTING TO THE ANTIGEN OF *Columba guinea*

ANTISERUM TO LIVIA	ABSORBED WITH CELLS OF	TITER OF AGGLUTINATION OF TEST CELLS			
		GUINEA (*CC*)	*CC*	*CC′*	LIVIA (*C′C′*)
	. . .	8	10	10	10
150S4	*CC′*	0	0	0	0
	CC	0	0	10	10

NOTE: The digits in tables 1 and 2 represent the number of doubling dilutions (that is, the highest dilution) of the antiserum and respective reagents at which agglutination was observed. Thus, the first dilution was 1:60; 1 = 60, 2 = 120, 3 = 240 . . . 10 = 30,720.

with guinea cells, due to cellular antigens held in common between guinea and livia, and at a dilution of 1:30,720 with the other cells. When this antiserum was absorbed with cells of the heterozygotes, *CC′*, antibodies were removed for each kind of test cell. As previously stated, absorption of antiserum to livia with homozygous CC cells results in a reagent for C′, specific for the cells of livia and the heterozygotes (*CC′*). This antiserum when absorbed by homozygous *CC* cells agglutinated heterozygous *CC′* and livia cells (presumably *C′C′*) to a dilution of 1:30,720. However, reagents for C′ were obtained from several other antisera to livia by parallel procedures but the titers of these reagents varied considerably.

In a total of 150 birds of 31 families involving only antigen C of guinea, all had either C or C′ or both. The proportions of birds heterozygous *CC′* and homozygous *C′C′* from matings of heterozygotes *CC′* to livia *C′C′*, and the proportions of heterozygotes *CC′* and homozygotes *CC* and *C′C′* from matings *inter se* of heterozygotes were in agreement with genetic expectation within these families. Further, the offspring of pen matings of

birds homozygous for C, either *inter se* or with other hybrids possessing either E or F of guinea, could be demonstrated to be homozygous *CC* or heterozygous *CC'* by virtue of their reactivities with the reagents for C and C', respectively. (The details of the genetic data will be supplied on request.) Also, cells from 105 livia and 43 back-cross hybrids not containing C were tested with one or more reagents for C' and all were reactive, as expected. The cells of these 43 back-cross hybrids were not agglutinated with the reagent for C, nor were those of livia which were so tested. Hence, the expectation is that C' is probably universally present in livia.

Other Substances of Livia Contrasting to the Specific Substances of Guinea.—It would be expected that, if livia possesses a substance C' in contrast to the C of guinea, it might also possess contrasting substances to A, B, E, and F of guinea. It should be possible to detect such substances, if they exist, by methods paralleling that outlined above for detecting C'.

TABLE 2

COMPARISON OF ANTIBODY CONTENT BY TITERS OF THREE REPRESENTATIVE ANTI-LIVIA SERA

ANTISERUM TO LIVIA	UNABSORBED TEST CELL, LIVIA	ABSORBING CELLS: *AA* TEST CELLS: *AA*	*AA* TEST CELLS: *AA'*	*AA* TEST CELLS: *A'A'* (LIVIA)	*BB* TEST CELLS: *BB*	*BB* TEST CELLS: *BB'*	*BB* TEST CELLS: *B'B'* (LIVIA)
150S4	10, 11	0	6	7	0	2	3
712F2	11	..[a]	..	..	0	0	0
717F3	9	..	..	..	0	0	1

ANTISERUM TO LIVIA	ABSORBING CELLS: *CC* TEST CELLS: *CC*	*CC* TEST CELLS: *CC'*	*CC* TEST CELLS: *C'C'* (LIVIA)	*EE* TEST CELLS: *EE*	*EE* TEST CELLS: *EE'*	*EE* TEST CELLS: *E'E'* (LIVIA)	*FF* TEST CELLS: *FF*	*FF* TEST CELLS: *FF'*	*FF* TEST CELLS: *F'F'* (LIVIA)
150S4	0	10	10	0	7	7	0	6	7
712F2	0	9	9	0	3	3	0	0	2
717F3	0	5	5	0	0	0	0	0	0

[a] Not tested.

There was available an initial group of 64 hybrids from matings *inter se* of back-cross hybrids possessing a specific antigen of guinea other than C. Birds homozygous for A, B, E, or F, respectively, were detected within these hybrids. The ratio of homozygotes to heterozygotes in these four kinds of progenies agreed well with genetic expectations.

When cells from homozygotes for any one antigen of guinea were used to absorb anti-livia serum, the resulting reagent was specific for the cells of both livia and the heterozygotes of the particular antigen of guinea. For example, it is shown in table 2 that anti-livia serum No. 150S4, absorbed with homozygous *AA* or *FF* cells would titer 6 or 7 doubling dilutions (from 1:60) with livia and heterozygous *AA'* or *FF'* cells. Further, the same antiserum absorbed with pooled homozygous *BB* cells resulted in a

reagent which would titer 2 or 3 doubling dilutions (from 1:60) with livia or heterozygous *BB'* cells, and when absorbed with pooled homozygous *EE* cells would titer 7 doubling dilutions (from 1:60) with livia or heterozygous *EE'* cells. These results can best be interpreted by assigning to the cells of livia the substances A', B', E', and F', antithetical, respectively, to A, B, E, and F of guinea. Other anti-livia sera varied considerably in their antibody content for these specific substances. A contrast of the titers of three representative antisera of the nine investigated, with appropriate absorptions for the detection of A', B', and C', E', and F', is given in table 2. No antiserum failed to contain antibodies for C', although the titers varied from 4 to 10 doubling dilutions from 1:60. Some of the anti-livia sera lacked antibodies for A', B', E', or F'. B' is apparently the weakest antigen and C' the strongest.

Discussion.—From matings of back-cross hybrids involving antigen C, 182 hybrids have been tested with both anti-C and anti-C'. Some hybrids had either C or C', and the others contained both C and C' and were heterozygotes. The other contrasting antigens of guinea and livia (A and A', B and B', E and E', F and F') have not been tested as completely as have C and C', since only a few homozygotes for each of these substances of guinea have been available at any one time. Nevertheless, 123 hybrids, in addition to those involving antigen C and C', were tested with the specific reagents for A and A', B and B', and E and E', or F and F', and the results have agreed with the hypothesis of a two-antigen system for each contrasted pair of antigenic characters. The possession by livia of other cellular antigens in contrast to guinea besides the five described in this paper is not excluded by the present study.

The method presented in this paper for distinguishing heterozygous blood types could be applied to blood group systems of other animals such as cattle, chickens, and possibly the human blood group systems. For example, in regard to the human ABO system, if immune sera produced in experimental animals against human cells carrying the O substance (AO or OO) were absorbed with homozygous *AA* cells, the resulting reagent might be specific for the O substance.

Summary.—Following matings *inter se* of back-cross hybrids resulting from matings to livia of hybrids between *C. guinea* and *C. livia*, hybrids were obtained which were homozygous for any one of five cellular antigens which distinguish guinea from livia, e.g., *AA*, *BB*, *CC*, *EE*, and *FF*. The basic assumption is that these respective homozygous birds would contain all the cellular antigens of livia except that antigen which was antithetical to a particular antigen of guinea, the genes for which were present in homozygous form. Absorption of anti-livia serum by the blood cells of respective homozygotes provided reagents for the detection of the contrasting substances of livia, e.g., A', B', C', E', and F'. The cells of

the birds in the back-cross population contained either an antigen specific to guinea or to livia or both. The general conclusion from these results is that the antigens A and A′, B and B′, C and C′, E and E′, and F and F′ are contrasting members of respective genetic systems.

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*INTERACTION BETWEEN ALLELES AFFECTING CELLULAR ANTIGENS FOLLOWING A SPECIES CROSS IN COLUMBIDAE**

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The majority of the cellular antigens now recognized in various animal species appear to be the more or less direct products of their causative genes. However, a few exceptions to this general rule have been noted in species hybrids in pigeons and doves[1, 2] and in ducks,[3] in which the blood cells of the species hybrids and some back-cross hybrids[4] have possessed new antigenic specificities which were not detectable in the respective parental species. These new specificities have been called "hybrid substances."

This paper deals with the detection of a hybrid substance in the cells of certain back-cross hybrids from the mating of *Columba livia* and *C. guinea*, and presents evidence that this hybrid substance is probably due to the interactions of allelic genes, peculiar to livia and to guinea, respectively.

Materials and Methods.—The techniques employed in this experiment with respect to the production of antibodies, and the absorptions to make specific reagents are essentially the same as those previously described.[2, 4, 5] The presence of agglutination of the various kinds of cells with specific reagents is the index of the presence of an antigenic substance, as contrasted to the absence of the antigenic substance if no agglutination is observed.

Following back-crosses to livia of the F_1 hybrids and selected back-cross hybrids from the mating of guinea and livia, a cellular antigen called C specific to guinea, has been obtained in unit form.[6] By matings *inter se* of these back-cross birds, heterozygous for the C substance, offspring were obtained which were homozygous for the C antigen. The homozygotes could be differentiated from the heterozygotes serologically and by progeny

tests as described.[5] Both homozygotes and heterozygotes were available for the tests. The antigenic substance in livia, antithetical to the C of guinea, has been called C′.[5] The C and C′ substances apparently form a closed antigenic system; that is, all the back-cross hybrids from matings to livia or *inter se* have been homozygous *CC*, homozygous *C′C′*, or heterozygous *CC′*.

Experimental Results.—Twelve antisera against the cells of the homozygotes (*CC*) were produced in rabbits. These antisera, when absorbed with an excess of the cells of livia (*C′C′*), provided reagents specific for the C substance. Consequently, they reacted with the cells of guinea, the homozygotes (*CC*), and the heterozygotes (*CC′*), but not at all with the cells of livia (*C′C′*). However, when absorbed with an excess of the cells of either the homozygotes (*CC*) or the heterozygotes (*CC′*) they were completely depleted of antibodies. All these anti-C reagents reacted at dilutions from 1:1920 to 1:15,360, as given in table 1.

TABLE 1

THE AGGLUTINATIONS OF THE ERYTHROCYTES OF LIVIA, GUINEA, AND OF BACK-CROSS BIRDS HOMOZYGOUS (*CC*) AND HETEROZYGOUS (*CC′*) FOR THE C ANTIGENS OF GUINEA, WITH REAGENTS PREPARED FROM ANTISERA TO THE C SUBSTANCE FROM HOMOZYGOTES AND HETEROZYGOTES

	THE RANGE OF REACTIONS OF 6 ANTI-*CC* SERA AFTER ABSORPTION WITH CELLS AS LISTED				THE RANGE OF REACTIONS OF 5 ANTI-*CC′* SERA (LACKING ANTIHYBRID ANTIBODIES) AFTER ABSORPTION WITH CELLS AS LISTED				
TEST CELLS	UNABSORBED	LIVIA (*C′C′*)	*CC*	*CC′*	UNABSORBED	LIVIA (*C′C′*)	*CC*	*CC′*	*CC* + LIVIA
Livia	8–13[a]	0	0	0	9–13	0	8	0	0
CC	9–12	6–9	0	0	8–13	6–11	0	0	0
CC′	8–13	6–8	0	0	9–13	6–11	9	0	0
Guinea	8–13	6–9	0	0	8–12	6–9	0	0	0

	THE RANGE OF REACTIONS OF 3 ANTI-*CC′* SERA AFTER ABSORPTION WITH CELLS AS LISTED					
TEST CELLS	UNABSORBED	LIVIA (*C′C′*)	*CC*	*CC′*	*CC* + LIVIA	GUINEA + LIVIA
Livia	8	0	4	0	0	0
CC	8	5	0	0	0	0
CC′	8	5	4	0	1–3	1–3
Guinea	7	4	0	0	0	0

[a] The numbers refer to titers based on doubling dilutions of the reagents, starting at 1:60; e.g. 1 = 1:60, 2 = 1:120, 3 = 1:240 . . . 13 = 1:245,760; 0 indicates no agglutination at the first dilution of the reagent.

Thirteen antisera were produced against the cells of the heterozygotes (*CC′*). These, after absorptions with the cells of livia, also reacted strongly with the cells of guinea, the homozygotes (*CC*) and the heterozygotes (*CC′*). They were completely depleted of antibodies following absorptions with the cells of the heterozygotes (*CC′*). However, unlike the anti-*CC* sera, following absorptions with an excess of the pooled cells of two or more ho-

mozygotes (*CC*), they became anti-C′ reagents which reacted strongly (1:7680 to 1:30,720) with the cells of livia and of the heterozygotes (*CC′*) but not at all with the cells of guinea or of the homozygotes (*CC*), as is given in table 1. This latter test clearly differentiated between the cells of the homozygotes (*CC*) and those of the heterozygotes (*CC′*). This differentiation is in complete accord with the differentiations based on progeny and pedigree tests as reported elsewhere.[5]

Of these 13 antisera produced against the cells of the heterozygotes (*CC′*), ten were completely depleted of antibodies when absorbed (*a*) with pooled cells of both parental species (livia and guinea) or (*b*) with cells of the homozygotes (*CC*) pooled with cells of livia. However, two antisera, similarly absorbed, would react weakly at the absorbing dilution (1:60) only with cells of the heterozygotes (*CC′*) and one antiserum (300F9), thus absorbed, reacted strongly (titer of 1:240) only with the cells of the heterozygotes (*CC′*).

This reaction was undoubtedly due to a new specificity peculiar to the cells of the heterozygotes (*CC′*). To minimize the possibility of individual differences among the birds giving rise to this phenomenon, the cells of 90 representatives of livia, of 55 homozygotes (*CC*), of 93 heterozygotes (*CC′*) and of 7 birds of guinea were tested with the reagent for this hybrid substance. The cells of all the heterozygotes (*CC′*) were strongly agglutinated by this reagent but none of the cells in the other three groups reacted with it. Furthermore, this reagent agglutinated strongly the cells of 35 offspring (*CC′*) of matings to livia of birds homozygous for C, even when the pooled cells of their respective parents were employed in absorptions for the preparation of the reagent.

As previously reported,[6] there are four other distinct antigenic substances, A, B, E, and F, particular to guinea as contrasted to livia. Following the same procedures as described above for the detection of the C substance, antithetical substances designated as A′, B′, E′, and F′ have been demonstrated in the cells of livia[5] and corresponding back-cross hybrids. Both homozygotes and heterozygotes for the gene or genes producing the respective substances have been recognized. It was therefore pertinent to determine whether the erythrocytes of the respective heterozygotes (*AA′*, *BB′*, *EE′*, and *FF′*) also possessed a hybrid substance. Repeated immunizations of rabbits were made with the cells of each of these heterozygote types, but to date no antibody specific to a hybrid substance has been demonstrated for the four kinds of heterozygotes.

The erythrocytes of Ring dove (*Streptopelia risoria*) are agglutinated by the reagent for the C of guinea, and therefore possess one or more "reactive groups" of the C substance, a C-like substance (unpublished data). The hybrids from matings of livia (*C′C′*) and Ring dove (presumably homozygous for the C-like substance) will be heterozygous for the genes pro-

ducing C′ and the C-like substance. Such hybrids possess a hybrid substance,[2] which may be, but need not be, related serologically to the interaction products of the heterozygotes *CC′*. The reagent for the *CC′* hybrid substance was tested at the absorbing dilution (1:60) with the cells of six hybrids from the mating of livia and Ring dove, and no agglutination was observed. These results indicate that, since the antibodies in the specific reagent are not reactive with the cells tested of hybrids between livia and Ring dove, the gene or genes in guinea, which interact with those of livia to effect a new antigenic specificity, are not found in Ring dove, at least not as homologs. That is, the gene or genes of guinea, associated with the C substance, which interact with one or more genes of livia to effect the new or changed antigenic specificity of the heterozygous *CC′* cells, are not present in Ring dove, although an antigenic substance, and therefore a causative gene, related to the C of guinea exists in Ring dove. The specificities of other antisera need to be examined for possible interrelationships of these two hybrid substances before concluding that an interrelationship does not exist.

The present evidence indicates that a hybrid substance is associated only with the *CC′* heterozygotes, not with the four other heterozygotes (*AA′*, *BB′*, *EE′*, or *FF′*). It might be argued that the new or changed antigenic specificity of the cells of the *CC′* birds was simply a change in specificity conferred upon the heterozygotes by some sort of rearrangement of the antigenic substances C and C′ when together. This might be called an "interaction product" of the antigens C and C′. Such an explanation appears much less tenable than if each of the five kinds of heterozygotes were characterized by a new antigenic specificity. Therefore a somewhat more plausible explanation appears to be that this hybrid substance of the *CC′* heterozygote is the result of genic interaction, but whether of alleles producing C and C′, respectively, or of other genes on the same chromosomes, is not yet determined.

Summary.—Following backcrosses to *C. livia* of the hybrids and selected back-cross hybrids from the mating of *C. guinea* and *C. livia*, a cellular antigen called C specific to guinea has been obtained in unit form. A contrasting substance called C′ has been demonstrated in livia. The C′ of livia and C of guinea are presumably produced by one or more pairs of allelic genes. Antisera were produced against the cells of the heterozygotes (*CC′*) which, when absorbed (*a*) with pooled cells of both parental species or (*b*) with the cells of the homozygotes (*CC*) pooled with cells of livia, provided reagents which reacted only with cells of the heterozygotes (*CC′*). These antisera therefore contained antibodies specific to an antigenic factor different from those found in either parental species or the homozygotes (*CC*).

Presumably this hybrid substance is the result of genic interaction, but

whether of alleles producing C and C′, respectively, or of other genes on the same chromosomes, is an open question.

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GENETIC EXCHANGE IN ESCHERICHIA COLI K12: EVIDENCE FOR THREE LINKAGE GROUPS

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The genetic analysis of recombination within strain K-12 of *Escherichia coli* has, until recently, been considered entirely in terms of the hypothesis of an orthodox sexual mechanism involving union of entire cells, followed by a normal meiotic cycle of chromosome pairing, crossing over, and reduction.[1] The evidence in favor of this assumption was many-sided and included both the existence of unstable strains which behaved like heterozygous diploids by segregating out stable recombinant strains, and the inability of cell-free filtrates (in contrast to type transformation in pneumococcus) to induce recombination. Since recombination is a rare event, it has not been possible to observe zygote formation and the evidence for a classical genetic mechanism has remained circumstantial.

While originally it was believed that zygote formation occurred between similar cells, it has recently been observed[2,3] that K-12 strains belong to two types (sexes) called F^+ and F^-, and that recombination occurs largely, if not entirely, between cells of opposite sex. Matings of $F^- \times F^-$ strains are completely sterile while $F^+ \times F^+$ matings are relatively infertile in comparison to $F^+ \times F^-$ matings. In addition, Hayes[2] has observed that while streptomycin kills (destroys the ability to form a colony) F^+ and F^- cells at equal rates, its effect on the fertility of the two sexes is markedly different. The fertility of F^- strains treated with streptomycin is lost much more rapidly than the fertility of similarly treated F^+ strains. Likewise the fertility of ultra-violet treated F^- strains is lost at a faster

rate than the fertility of ultra-violet treated F^+ strains. In fact, low doses of ultra-violet light may increase the fertility of F^+ strains by a factor of 10. To explain these results, Hayes has suggested that genetic exchange is unidirectional and that F^+ cells act as gene donors while the F^- cells are gene acceptors. In support of this hypothesis is the additional observation[2,3] that the genetic contribution of the F^+ parent is on the average much less than that of the F^- parent, a result which suggests that only part of the genetic material of the F^+ parent is effectively transferred to the zygote, the remainder either being lost during formation of the F^+ gamete or eliminated during reduction.

The finding that the two sexes make unequal genetic contributions (which we shall call F^+ polarity) must naturally have an effect on genetic analysis, for the previously deduced linkage relationships might have been unconnected with true linkage to linear chromosomes but might instead have been the consequence of polarity. We have, therefore, examined in greater detail the effect of polarity reversal on genetic segregation. This analysis will utilize to a large extent the results of some of our recent genetic crosses, though to a lesser degree we shall also consider some of the published results of the Lederbergs, Rothfels, and Cavalli.

The analysis consists of two sections, the first of which examines the theoretical consequences of the transfer of incomplete portions of the F^+ genome to the zygote and deals with the results to be expected if each F^+ cell contains several non-homologous chromosomes of which only one is usually transferred to the zygote. The second section considers the experimental results in the light of the theoretical treatment and concludes that the present data are best interpreted on the basis of three independent linkage groups. Only the genetic aspects of recombination, and not the mechanism by which the F^+ genetic contribution is transferred to the zygote, will be considered.

Theory.—We must first consider the manner in which genetic crosses in K-12 are made. Recombination is a very rare event and a special technique must be used to separate the recombinant cells from the unrecombined parental cells. This is accomplished, following the original example of Tatum and Lederberg,[4] by mixing in minimal agar two mutant strains having complementary nutritional requirements. Under these conditions, the parental cells cannot grow and only recombinant cells possessing the wild type alleles can form a colony. The nutritionally non-exacting recombinant cells have been called prototrophs[5] since they resemble the wild type cells in having acquired the inheritable capacity to synthesize all of the growth factors upon which the mutant strains were dependent. We shall extend the definition of prototroph to include all recombinant cells found either on minimal agar alone or on minimal agar supplemented with one or more growth factors.

In addition to nutritional requirements the parental strains are usually labeled with a number of characters (markers) which are *not selected* by growth in minimal medium. Such characters include the ability to ferment specific sugars and the resistance or sensitivity to various phages or antibiotics. It is generally assumed that both the nutritional selective markers and the unselective markers have arisen by single mutational steps, but this supposition has only rarely been tested. Prototrophs may display patterns of the unselective markers which are different from those of either of the parental strains and thus furnish material for genetic analysis. It is essential to distinguish the behavior of the selective markers from that of the unselected markers and to remember that the experimental methods restrict the observed recombinants (prototrophs) to a non-random sample of the total recombinant cells.

We shall now develop the genetic results to be expected if the F^+ parent contributes only a fraction of its genetic material to the zygote. In this case, the zygote will not be a complete diploid and following reduction both viable and inviable cells will be formed since an incomplete genetic complement will be present within many of the reduced cells. If the parental cells contain only one chromosome, the incomplete transfer must involve the elimination of part(s) of the single chromosome and the production of chromosomal fragments. These fragments, by themselves, would tend to be inviable and, in general, for viable recombinants to be formed, double crossing over would have to occur. On the other hand, if several non-homologous chromosomes are present, the incomplete transfer could be in the form of intact chromosomes which by themselves could show normal chromosome behavior. In view of the comparative simplicity of this latter possibility, we shall develop it first, indicating where relevant in what respects it may be expected to differ from the former scheme. We shall assume that: (1) Each haploid nucleus contains several non-homologous chromosomes. (2) Zygotes are usually formed from the union of only one of the F^+ chromosomes with the full complement of F^- chromosomes. Less frequently union occurs between two or more of the F^+ chromosomes and the full F^- complement. (3) Pairing occurs between homologous chromosomes with reduction occurring following crossing over.

These assumptions lead to the following predictions:

(1) Recombinants will tend to derive most of their genetic material from the F^- parent.

(2) Two types of linkage will be observed:

(a) between genes on the same chromosome; their recombination values should lead to a linear arrangement.

(b) between genes on non-homologous chromosomes; this linkage results from the incomplete participation of the F^+ genome and will be

between the genes on the unrecombined chromosome(s), and the selective marker(s) of the F^- parent. We should distinguish the following situations:

(*i*) *The selective markers of the parental strains located on homologous chromosomes.* In this case, we select for recombination between only one of the homologous pairs of chromosomes and thus the recombinants will tend to derive their remaining chromosomes from the F^- parent. Recombinants for the unselected chromosomes result from the union of more than one F^+ chromosome with the F^- chromosomes. The linkage values will be equal between all of the loci on unselected chromosomes and the selective marker(s) of the F^- parent. Thus, if we were to assume the existence of only one chromosome we would be forced to postulate a multi-armed chromosome. Reversal of polarity between the parental strains should result in a corresponding reversal of the linkage between the loci on the unselected chromosomes and the selective markers. In contrast, no change should be observed in the segregation behavior of the loci on the selected chromosome.

(*ii*) *The selective markers of the F^+ strain located on a non-homologous chromosome to that containing the F^- selective markers.* For a viable recombinant to occur, pairing must take place between the chromosome containing the F^+ selective markers and the corresponding F^- chromosome. The remaining chromosomes will tend to be derived from the F^- parent, and their loci will tend to show linkage with the selective markers of the F^- parent. Reversal of polarity will affect the recombination frequency of loci on both chromosomes with the largest recombination values always occurring between the genes in the chromosome containing the selective markers of the F^+ parent.

The theory is simply extended to situations in which each parental strain contains selective markers on more than one chromosome. In these cases, viable recombinants will be produced only when more than one F^+ chromosome is contributed to the zygote and we should correspondingly expect a greatly decreased frequency of viable recombinants. It is also obvious that when recombinants for a given chromosome occur their segregation pattern, in general, will not be random but instead be strongly influenced by selection for the wild type alleles of the selective markers. The following cases might usefully be distinguished.

(1) Recombination within a chromosome pair in which a selective marker is located in only one of the two homologs. All recombinants will contain the selective marker and, to a variable extent, genes linked to it. Assuming random crossing over the frequency of alleles belonging to the strand containing the selective marker should vary from 100% for closely linked genes to 50% for relatively unlinked genes on the same chromosome. (2) Recombination within a chromosome pair in which selective

markers are located on both homologs. In this case, the frequency of F^+ (or F^-) alleles may range from 0 to 100% depending upon exact linkage relationships. This case will obviously be more difficult to analyze than the preceding one. (3) Recombination within a chromosome pair lacking selective markers. Though in general this situation will be rare, its analysis is straightforward since the frequency of the F^+ and F^- alleles will be equal and ordinary mendelian analysis should apply. Recombinants of this type can be studied by selecting for analysis, prototrophs containing F^+ alleles of unselected chromosomes.

Materials and Methods.—Most of the experiments to be discussed in this paper deal with crosses between two complementary bacterial strains derived from K-12. They are 58-161, a methionine requiring (M^-) mutant, which ferments lactose, maltose, mannitol, galactose and xylose, is sensitive to phage T1 and is resistant to phage T3 (Lac^+, Mal^+, $Mann^+$, Gal^+, Xyl^+, V_1^s, V_3^r). This mutant was originally described as biotin dependent (B^-), but in recent years it has grown well without biotin and is presumed to have lost this requirement. The second strain is W677, a mutant requiring threonine, leucine, and vitamin B_1 ($T^- L^- B_1^-$) and whose unselected markers are complementary to 58-161, that is, Lac^-, Mal^-, $Mann^-$, Gal^-, Xyl^-, V_1^r, V_3^s. Each of these strains, as desired, can be made resistant to azide (Az^r) or to streptomycin (S^r). In some experiments by Cavalli, two additional K-12 strains and their derivatives have been employed. They are Y10 which is ($T^- L^- B_1^-$), and W945 which in addition to being ($T^- L^- B_1^-$) is negative also for lactose, maltose, xylose, and galactose (Lac^-, Mal^-, Xyl^-, Gal^-). From both Y10 and W945 two types of mutational reversions were obtained, ($T^+ L^+ B_1^+$) and ($T^+ L^+ B_1^+ S^r$) so that ($T^- L^- B_1^- S^r$) from Y10 could be crossed to ($T^+ L^+ B_1^+ S^s$) of W945 and vice versa. Allelism tests of W945 and W677 indicated (Cavalli personal communication) that Lac^- and Xyl^- are allelic while Gal^- and probably Mal^- are not allelic. In most previously published experiments, 58-161 behaved as the F^+ strain while W677, Y10, and W945 behaved as F^- strains. An F^- strain of 58-161 was recently obtained by Hayes and since F^- strains can be converted to F^+ strains by infection,[2,3] it is now possible to cross these strains under reversed polarity conditions.

The nutrient requirements, M, L, T, B_1, and Lac, as well as resistance to azide and streptomycin have been used as selective markers. For a description of the experimental procedure, the reader is referred to the original publications of the Lederbergs, Cavalli, and Hayes. The marker characteristics of prototrophs were largely scored by examination of entire prototroph colonies though in some crosses by Hayes, the progeny of single colonies isolated from prototrophs were examined.

Experiments.—The experiments we now wish to report deal with the

effect upon genetic segregation of polarity reversal. We shall present both the results of crosses in which the F^+ parent was strain 58-161 and the F^- parent was strain W677, and of the complementary cross in which W677 was the F^+ parent and 58-161 the F^- parent. We shall consider the results in the light of the general theory according to which the decreased genetic yield of the F^+ alleles is a consequence of an incomplete transfer of the F^+ chromosomal complement to the zygote. We should emphasize that owing to the experimental method prototrophs are limited to those recombinants which contain all of the selective markers. Under the general theory, this leads to the consequence that, in general, recombination will only be observed between loci belonging to the same chromosome(s) as the selective marker(s) of the F^+ parent, though in rarer cases (as we shall show further on) unselected chromosomes must be transferred to the zygote. We shall take the appearance of an unselected F^+ allele in a prototroph as an indication of linkage to an F^+ selective marker. On the contrary the infrequent occurrence of an F^+ allele does not necessarily imply a lack of linkage with F^+ selective marker(s), but instead may sometimes be due to still closer linkage with an F^- selective marker.

Our analysis will thus start by considering the correlation between the use of specific selective markers and the occurrence of specific F^+ alleles within prototrophs. Three groups of linked genes are indicated by this method of analysis. The existence of the first group is suggested by crosses between 58-161 F^- and W677 F^+ in which methionine is the F^+ selective marker. The results of these crosses are shown in table 1 and demonstrate that only one F^+ allele, vitamin B_1, appears at all frequently in the prototrophs. It thus would appear that the only unselected marker which is linked to methionine is B_1. A second group of linked genes is revealed by the reciprocal cross, 58-161 $F^+ \times$ W677 F^-, the results of which are also shown in table 1. In these crosses, in which threonine and leucine are the F^+ selective markers, three of the unselective markers, Az, V_1, and Lac, appear frequently in the prototrophs and must be linked to either T or L. Unfortunately, we cannot distinguish the effect of selecting for threonine against that of selecting for leucine since the results from singly supplemented crosses have been ambiguous. For simplicity we shall consider (TL) as a single selective marker, reserving final judgment until more conclusive evidence has been obtained. The remaining four unselective markers, S, V_3, Mal, and Mann, are not selected for by either (TL) or M, a fact which suggests the presence of one or more additional linkage groups.

This conjecture is supported by two series of experiments. The first of these is reported by Rothfels[6] who crossed a streptomycin resistant F^+ strain of 58-161 with a streptomycin sensitive F^- strain of W677 on minimal agar supplemented with streptomycin, B_1, threonine, and leucine. The

TABLE 1

THE EFFECT OF POLARITY REVERSAL UPON GENETIC SEGREGATION

58-161 = $Lac^+ Mal^+ Mann^+ V_1^s V_3^r S^r Az^r T^+ L^+ B_1^+ M^-$
W677 = $Lac^- Mal^- Mann^- V_1^r V_3^s S^s Az^s T^- L^- B_1^- M^+$
Crosses #1 and #3 = 58-161/F^- × W677 F^+ on minimal agar + B_1
Crosses #2 and #4 = 58-161/F^+ × W677 F^- on minimal agar + B_1

	SELECTIVE MARKERS	UNSELECTIVE MARKERS
Crosses #1 and #2	T, L, M	Lac, Mal, S, Az, B_1
Crosses #3 and #4	T, L, M	Lac, Mann, V_1, V_3, S

LAC	MAL	S	Az	B_1	CROSS #1	CROSS #2	LAC	MANN	V_1	V_3	S	CROSS #3	CROSS #4
+	+	r	r	+	106	..	+	+	s	r	r	24	2
+	+	r	r	−	145	..	+	+	s	r	s	..	..
+	+	r	s	+	..	..	+	+	s	s	r	..	1
+	+	r	s	−	5	..	+	+	s	s	s	..	..
+	+	s	r	+	1	..	+	+	r	r	r	3	..
+	+	s	r	−	1	..	+	+	r	r	s	..	..
+	+	s	s	+	..	..	+	+	r	s	r	..	..
+	+	s	s	−	2	..	+	+	r	s	s	..	..
+	−	r	r	+	3	..	+	−	s	r	r	6	1
+	−	r	r	−	6	..	+	−	s	r	s	..	2
+	−	r	s	+	..	..	+	−	s	s	r	..	1
+	−	r	s	−	..	1	+	−	s	s	s	1	20
+	−	s	r	+	2	5	+	−	r	r	r	..	..
+	−	s	r	−	8	64	+	−	r	r	s	..	..
+	−	s	s	+	4	..	+	−	r	s	r	..	..
+	−	s	s	−	10	4	+	−	r	s	s	..	2
−	+	r	r	+	1	..	−	+	s	r	r	..	..
−	+	r	r	−	4	..	−	+	s	r	s	..	..
−	+	r	s	+	..	..	−	+	s	s	r	..	..
−	+	r	s	−	..	1	−	+	s	s	s	..	..
−	+	s	r	+	..	..	−	+	r	r	r	..	..
−	+	s	r	−	..	1	−	+	r	r	s	..	..
−	+	s	s	+	..	..	−	+	r	s	r	..	..
−	+	s	s	−	..	..	−	+	r	s	s	..	1
−	−	r	r	+	..	..	−	−	s	r	r	..	..
−	−	r	r	−	1	1	−	−	s	r	s	..	..
−	−	r	s	+	..	..	−	−	s	s	r	..	1
−	−	r	s	−	..	..	−	−	s	s	s	..	47
−	−	s	r	+	..	11	−	−	r	r	r	..	..
−	−	s	r	−	..	169	−	−	r	r	s	..	..
−	−	s	s	+	..	..	−	−	r	s	r	..	..
−	−	s	s	−	1	43	−	−	r	s	s	..	28
					300	300						34	106

TABLE 2

THE EFFECT OF STREPTOMYCIN SELECTION UPON GENETIC SEGREGATION

(From Rothfels[6])

58-161 = $Lac^+ V_1^s S^r T^+ L^+ B_1^+$
W677 = $Lac^- V_1^r S^s T^- L^- B_1^-$
Cross #1 = 58-161/F^+ × W677/F^- on minimal agar + B_1
Cross #2 = 58-161/F^+ × W677/F^- on minimal agar + B_1 + streptomycin + threonine + leucine

	CROSS #1	CROSS #2
V_1^s Lac^+	407	66
V_1^s Lac^-	497	111
V_1^r Lac^+	39	14
V_1^r Lac^-	267	1189
	1210	1380

TABLE 3

GENETIC SEGREGATION FOLLOWING STREPTOMYCIN SELECTION

(From Cavalli[3])

W945 = $Lac^- Gal^- Mal^- Xyl^-$
Y10 = $Lac^+ Gal^+ Mal^+ Xyl^+$
Cross #1 = W945/$F^+/S^r/T^- L^- B_1^-$ × Y10/$F^-/S^s/T^+ L^+ B_1^+$
Cross #2 = Y10/$F^+/S^r/T^- L^- B_1^-$ × W945/$F^-/S^s/T^+ L^+ B_1^+$

MAL	XYL	LAC	GAL	CROSS #1	CROSS #2
+	+	+	+	175	...
+	+	+	−	...	2
+	+	−	+	1	...
+	+	−	−	1	181
+	−	+	+	14	...
+	−	+	−	1	...
+	−	−	+	...	...
+	−	−	−	1	140
−	+	+	+	253	2
−	+	+	−	4	...
−	+	−	+	3	...
−	+	−	−	1	14
−	−	+	+	276	2
−	−	+	−	8	...
−	−	−	+	4	...
−	−	−	−	26	156
				768	497

F^+ selective marker in this cross is streptomycin resistance since supplementation with threonine and leucine removes the selective action of these loci. If our theory is correct, we should find a greatly reduced proportion of the F^+ loci, Lac^+, and V_1^s when compared to crosses in which (TL) is selected. The results of this cross which are shown in table 2 support the theory since the number of prototrophs containing Lac^+ and/or V_1^s is seen to drop from 78% when (TL) is used as the selective marker to 15% when in their place resistance to streptomycin is selected. Since V_1 and Lac were the only unselective markers followed, these experiments do not tell us whether any additional markers tend to be transferred with streptomycin resistance. Fortunately some recent experiments of Cavalli[3] enable us to answer this question. Two types of crosses were made on minimal agar, supplemented with streptomycin. The first involved a mating between a F^- ($T^+ L^+ B_1^+$) streptomycin sensitive derivative of W945 with a F^+ ($T^- L^- B_1^-$) streptomycin resistant derivative of Y10. The second cross was nearly identical except that in this case, the F^- ($T^+ L^+ B_1^+$) parent was a streptomycin sensitive derivative of Y10. Selection occurs in both of these crosses for the S locus of the F^+ parent and the (TLB_1) loci of the F^- parent. The results of these crosses are shown in table 3 and indicate a high frequency for two of the unselective F^+ loci, Mal and Xyl. In contrast the F^+ loci, Lac and Gal, are present in small percentages.

We are thus in a position to draw the following provisional conclusions: (1) The decreased genetic contribution of the F^+ parent is not due to repeated elimination of a *specific* fraction of the F^+ genome. In fact, all the F^+ loci have an equal, though small, probability of being transferred to the zygote. (2) The F^+ loci are not transferred independently of each other, but in groups, that is, they show linkage with each other. We cannot as yet, however, conclude that these groups of linked genes are equivalent to chromosomes and thus of identical composition in all crosses. The data are also compatible with the hypothesis of a single F^+ chromosome which breaks randomly to pieces during every cross. To distinguish between these possibilities we must see whether the same linkage groups are obtained from crosses using different selective markers. Up till now, only several very preliminary crosses have been made with different selective markers. One of these[7] involved the use of azide resistance as the F^+ selective marker and streptomycin resistance as the F^- selective marker. Since the previous experiments have shown Az to be closely linked to (TL) we might expect the results of this cross to be very similar to the original 58-161 $F^+ \times$ W677 F^- cross. In fact, this is the result obtained as the F^+ alleles V_1^s and Lac^+, were frequently transferred to the doubly resistant recombinants in contrast to infrequent transfer of the F^+ loci, B_1, M, Mal, and Mann, loci which we have earlier shown to be unlinked to (TL).

In some of our crosses between 58-161/F^+ and W677/F^-, as well as in the reversed 58-161/F^- × W677/F^+ cross, B_1 has been used as a selective marker in conjunction with simultaneous selection for (TL) and M. No effect on segregation ratios was observed in either cross, although B_1 selection resulted in a reduction in the prototroph count. While this reduction amounted to tenfold in the 58-161/F^+ × W677/F^- cross, only a twofold reduction occurred in the reversed cross. These results agree with those in which B_1 is scored as an unselective marker and which indicated linkage of B_1 with M but not with any other marker tested. When M is employed as a selective marker, the only observable consequence of B_1 selection should be to decrease the number of prototrophs, an effect which should be much more noticeable when B_1^+ is in the F^+ parent, necessitating the transfer of an additional portion of the F^+ genome to the zygote.

Discussion.—In the foregoing sections, we have presented a series of experiments which indicate that genetic recombination in K-12 is in general limited to a fraction of the genetic loci, a result which we believe is due to an incomplete transfer of the F^+ loci to the zygote. Our discussion should consider both (1) the form of the F^+ contribution to the zygote and (2) the nature of the effective elimination of the untransferred fraction.

It appears to us most likely that the F^+ contribution occurs in the form of discrete chromosomes and not in the form of chromosomal fragments randomly formed during recombination. If this conjucture is true, we should always find the same linkage groups, independent of which selective markers are employed. As far as they go, our experiments support this belief, though it is obvious that the number of selective markers must be multiplied many-fold before this point can be established. We should note that the Lac locus does not behave exactly as predicted by our theory. In crosses in which Lac is linked to the F^+ selective marker, but not to the F^- selective marker, the frequency of the F^+ Lac allele is about 30%, significantly less than the expected $\geq 50\%$. At this preliminary stage in genetic analysis of K-12, it is possible to imagine many genetic complications such as intra-chromosomal interference or suppressor loci which would both account for the low Lac frequency and at the same time be in agreement with the general theory. It occurs to us that the low Lac frequency may be connected with the initial presence within 58-161 of a biotin requirement. This requirement has now disappeared, a result generally attributed to a back mutation. If, however, this reversion was due to a suppressor mutation at a different locus, we might observe that a biotin requirement would reappear in some recombinants. It is possible that the aberrant behavior of the Lac locus may be due to a linkage of Lac with the original biotin requirement.

The mechanism governing the infrequent appearance within prototrophs of unselected F^+ loci is, as yet, badly understood. Two facts, however,

seem clear: (1) These prototrophs cannot be due entirely to polarity reversal during a cross.[3] so that the F^+ strain behaves as F^- and the F^- strain behaves as F^+. If this were true, we might expect new recombinant classes involving loci linked to the F^- selective markers as these would, following polarity reversal, be linked to F^+ selective markers. We find, however, that loci unlinked to either the F^+ or F^- selective markers also exhibit recombination and must occasionally be transferred to the zygote; (2) K-12 strains differ among themselves in the frequency with which they transfer unselected loci. Only 2–3% of the prototrophs in crosses of 58-161 $F^+ \times$ W677 F^- contain unselected loci while approximately 20% of the prototrophs from crosses between W677 F^+ and 58-161 F^- contain unselected loci. Similar strain differences have been noted by Cavalli (personal communication).

In conclusion we should mention that we have not attempted to analyze linkage relationships within a linkage group as we feel that this analysis should await the completion of more comprehensive experiments involving a large number of loci. It is obvious that our assignment of the present loci to 3 linkage groups is provisional as the number may be increased when additional loci are considered. Finally it should be emphasized that our analysis in no way permits us to distinguish whether recombination involves the fusion of intact cells or is mediated by temperate bacteriophage particles as has been observed in *Salmonella typhimurium* by Zinder and Lederberg.[8]

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EFFECTS OF TEMPERATURE ON SPONTANEOUS AND INDUCED MUTATIONS IN ESCHERICHIA COLI*

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The frequency of spontaneous mutations in Drosophila has been shown to increase, in general, with increasing temperature, and the temperature coefficients reported range from about 2 to 5.[1] These findings played an important part in the early definition of mutation as a definite molecular rearrangement.[2] Very little is known of the response to post-treatment temperature of mutations induced by radiation or chemicals. Two kinds of effects could be investigated in this connection: the effect of temperature on the frequency of induced mutations, and the effect of temperature on the pattern of delayed appearance of induced mutations. In the latter category, Auerbach[3] has described an increase in the frequency of mustard-induced mosaics at low temperatures, which she ascribes to the stabilization by cold of metastable genic configurations induced by the mutagen, leading to extended delay in the shift to the stable mutant condition. Similar effects of temperature on the delayed action of mutagens in Neurospora and Aspergillis found in unpublished experiments by the authors are mentioned by McElroy and Swanson,[4] in support of the concept of mutation via metastable intermediates.

The hereditary change from sensitivity to resistance to bacteriophage in *Escherichia coli* can be followed with a degree of quantitative precision and technical ease that makes it a promising material for an investigation of temperature effects on spontaneous and induced mutation. This report is a preliminary account of such a study.

MATERIAL AND METHODS

Cultures of strain B/r of *Escherichia coli* were grown in a synthetic medium known as "A",[5] with aeration, for 18–24 hours, diluted with saline to an approximate titer of 10^8 cells per ml., and irradiated with 800 ergs per $mm.^2$ of ultra-violet light. Irradiation was carried out at room temperature, using a G. E. germicidal lamp, with mechanical agitation during the exposure. Yellow light was used to illuminate all operations following the ultra-violet treatment. After irradiation, aliquots of the treated suspensions were plated on nutrient agar plates and incubated at 37°, 25°, or 16°C. At various intervals of time, sets of six plates were withdrawn from the incubator and chilled rapidly in the freezing compartment of a refrigerator to arrest growth. Two of the six plates were washed with 10 ml. of

saline and the wash fluid was assayed, to determine the total number of bacteria on the plates; the four remaining plates were sprayed with an aerosol of a suspension of bacteriophage T1 having a titer of at least 10^{10} particles per ml., to ascertain the number of phenotypically demonstrable clones of bacteriophage-resistant mutants. The time intervals for withdrawal of plates from the incubator for washing and spraying were based upon previously established growth curves of the irradiated populations at

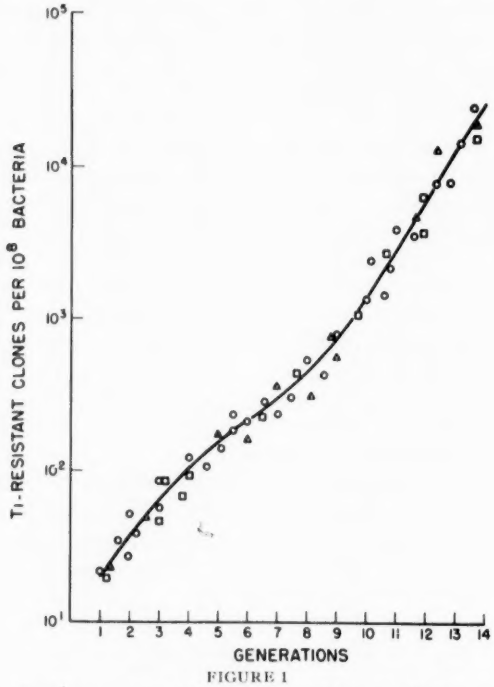

FIGURE 1

Spontaneous mutations to T1 resistance. Circles = incubated at 37°C. Squares = incubated at 25°C. Triangles = incubated at 16°C. Ordinates refer to T1 resistant clones per 10^8 bacteria *initially plated*.

the temperatures used, and were designed to cover the range from 0 to 14 generations. The data obtained from numerous experiments of this type permitted the construction of "expression curves" for the three temperatures studied, in which the number of T1-resistant clones per 10^8 irradiated cells was plotted against the number of post-irradiation generations. These curves are a measure of the pattern and extent of delayed appearance of the induced mutations—the final yield of induced mutations and the

number of generations required to achieve its complete manifestation being easily discerned by the leveling-off point. The necessary correction for spontaneous mutations was determined by similar experiments with unirradiated controls.

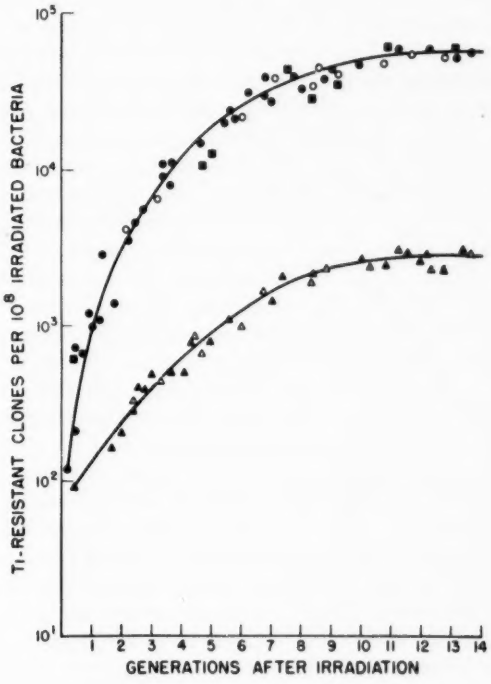

FIGURE 2

Effect of temperature on ultraviolet-induced T1-resistant mutants. Closed circles = incubated at 37° after irradiation. Closed squares = incubated at 25° after irradiation. Closed triangles = incubated at 16° after irradiation. Open circles = first postirradiation generation at 37°, all others at 16°. Open triangles = first postirradiation generation at 16°, all others at 37°. Ultraviolet dose, 800 ergs/mm.^{2}. Survival, 10 per cent.

RESULTS

1. Effect of Temperature on Generation Time.—The rate of growth of untreated and irradiated bacteria on nutrient agar plates was found to have a temperature coefficient of about 3. The generation time is twenty minutes at 37°, one hour at 25°, and three hours and twenty minutes at 16°

The lag phase for untreated cells is one hour and ten minutes at 37°, three hours and a half at 25°, and ten hours and forty minutes at 16°. For irradiated bacteria, the lag phases for the three temperatures are three hours, ten hours, and thirty hours for 37°, 25°, and 16°, respectively.

2. Effect of Temperature on Spontaneous Mutation Rate.—Figure 1 shows the number of T1-resistant clones, per 10^8 unirradiated cells plated, as a function of generations at 37°, 25°, and 16°C. The identity of the three curves shows that the process of spontaneous mutation to T1 resistance, expressed as the probability of mutation per bacterium per unit time, has the same response to temperature as does the generation time (Q_{10} *ca.* 3). The probability of mutation per bacterium *per generation*, which is the conventional way of expressing spontaneous mutation rate in bacteria, thus remains quite constant over the temperature range investigated. The data for spontaneous mutations are plotted in this rather unorthodox fashion so as to show most directly the magnitude of the corrections used in the preparation of expression curves for induced mutations. The same data can be used to calculate mutation rate according to standard formulae.

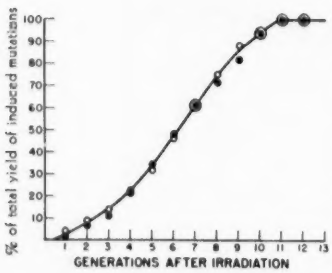

FIGURE 3

Effect of temperature on pattern of delayed appearance of ultraviolet-induced T1-resistant mutants. Open circles = postirradiation growth at 37° C. Closed circles = postirradiation growth at 16° C.

3. Effect of Temperature on Induced Mutations.—The response to temperature of induced mutation to T1 resistance is best shown by expression curves in which the number of detectable mutant clones per 10^8 irradiated cells is plotted against post-irradiation divisions at 37°, 25°, and 16°. These curves are shown in figure 2, already corrected for spontaneous mutations.

(*a*) *Effect of Temperature on the Final Yield of Induced Mutations:* The curve for bacteria incubated at 37° levels off at about 56,000 mutant clones per 10^8 irradiated cells; at 16°, the end-point number is about 2800, or 5 per cent of the yield at 37°. Thus, a profound effect of temperature on the yield of induced mutations is indicated, but the data obtained at 25° show that this effect cannot be expressed as a simple temperature coefficient. Although the data for bacteria incubated at 25° are fairly limited, there seems to be little, if any, reduction of the number of induced mutations at this temperature as compared with the yield at 37°. The temperature effect on the final yield of induced mutations is much greater between 25° and 16° than between 37° and 25°.

(*b*) *Effect of Temperature on the Pattern of Delayed Appearance of In-*

duced Mutants: Figure 3 shows percentages of the final yield of induced mutations as a function of post-irradiation divisions at 37° and at 16°. The identity of the two curves shows that, despite the large differences in final yield of induced mutations at the two temperatures, the span of generations required for a given fraction of the total to manifest itself is not modified by temperature. This supports the conclusion of Demerec and Cahn[6] that the pattern of delayed appearance is a characteristic and stable feature of a particular mutation.

(*c*) *The Temperature-Sensitive Period:* In the experiments described above, the irradiated cells were incubated at 37°, 25°, or 16° throughout the period of post-irradiation growth. Another series of experiments was conducted in which the irradiated bacteria were incubated at 37° for one generation, after which the plates were transferred to 16° for the duration of the experiment. Reciprocal experiments were also made, in which the first division was at 16°, and the remaining divisions took place at 37°. The data of these experiments, plotted also in figure 3, show clearly that the yield of induced mutations depends entirely on the temperature of incubation during the first post-irradiation generation. If the first division proceeds at 16°C., the final yield of mutation is 2800 per 10^8 treated bacteria, whether the remaining divisions take place at 16° or at 37°; similarly, if the temperature during the first division is 37°, the end-point number is 56,000 whether the subsequent divisions are at 37° or at 16°. Although 10–12 generations are required to achieve complete manifestation of the end-point number, it is the temperature during the first division that determines the outcome.

In an attempt to define the temperature-sensitive period more precisely, irradiated cells were exposed to one temperature for various fractions of the time required for the first generation before the plates were transferred to the other temperature. Intermediate yields were obtained, the initial temperature exerting an effect proportional to the fraction of the first generation passed under its influence. At present, these results can be accounted for equally well by two hypotheses concerning the temperature-sensitive period: (1) that the critical period is confined to the actual division process, the intermediate yields being a result of mixtures of cells that have and have not completed the first division; or (2) that the temperature effect is exerted gradually throughout the entire lag phase and first division.

(*d*) *Effect of Temperature after the First Post-irradiation Generation:* It was pointed out above that if the irradiated cells are allowed to pass through the first division at a given temperature, the temperature during the subsequent generations has no further effect on the course of the expression curve. Thus, after the first generation, the rate of appearance of mutant clones as a function of time has the same temperature coefficient as the processes of cell division and spontaneous mutation, about 3, so that the

rate of appearance of mutant clones per generation is not affected by temperature.

(*e*) *The Dose-Reduction Equivalent of the Temperature Effect:* The expression curve obtained at 16° after a dose of 800 ergs per mm.^{2} can be duplicated at 37° quite exactly when the dose is reduced to 100 ergs per mm.^{2}

DISCUSSION

These experiments have shown that, following treatment with ultra-violet radiation, there is a critical period up to and possibly including the first post-treatment division, during which temperature can influence profoundly the final yield of induced T1-resistant mutants. This observation supports the current view that the mutagenic action of ultra-violet light is indirect, and cannot be regarded as a simple photochemical effect. Beyond this, little can be deduced as to the nature of the genetic events following irradiation. Equally cogent arguments can be mustered in favor of three possible modes of action of ultra-violet light: (1) the production of intracellular mutagens; (2) the production of metastable genic states, and (3) the activation of systems of instability such as those described by McClintock.[7] Further work is required to make possible a critical analysis of these hypotheses. It is of particular importance to determine whether the temperature effects observed here are characteristic of mutational systems other than resistance to phage T1.

Another interesting feature of the results is the identity of the temperature coefficients of three presumably distinct processes—spontaneous mutation as a function of time, cell division, and the delayed appearance of induced mutations per unit time. This observation suggests that spontaneous mutation and the delayed appearance of induced mutation may be similar phenomena, and that both are intimately associated with the mitotic cycle. The separability of the processes of spontaneous mutation and cell division, however, has been demonstrated by studies with the chemostat,[8] and must be taken into account.

SUMMARY

(1) The final yield of ultra-violet-induced T1-resistant mutants in *E. coli* is reduced to about 5% when the incubation temperature following irradiation is 16°C., as compared with the result at 37°. There is little, if any, reduction at 25°. (2) The temperature effect on the number of induced mutants is confined to the period of the first post-treatment division. (3) The temperature coefficients of spontaneous mutation per unit time, cell division, and delayed appearance of induced mutations per unit time are identical (Q_{10} = *ca.* 3). Thus, spontaneous mutation per generation and delayed appearance of induced mutations per generation are constant over

the temperature range investigated. (4) The effect of post-treatment incubation at 16°C. can be duplicated at 37° by reduction of the ultraviolet dose from 800 to 100 ergs per mm.[2]

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ON THE EIGENFUNCTIONS AND EIGENVALUES OF THE GENERAL LINEAR ELLIPTIC DIFFERENTIAL OPERATOR

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Let K be a linear elliptic differential operator of order $2m (m \geq 1)$ with suitably differentiable coefficients on the bounded domain D of Euclidean n-space. During the past year, the author presented in several notes in these PROCEEDINGS a general method of solving boundary value problems for K on D.[1] In particular a number of results were obtained concerning the eigenfunctions and eigenvalues of K on D, including the completeness of the eigenfunctions if K is self-adjoint.[2] It is the principal object of this note to outline the proof that completeness persists even if K is not self-adjoint, provided that one admits higher order eigenfunctions into consideration (as in the case of linear transformations of finite dimensional complex vector spaces).[3] The existence of an infinite set of distinct complex eigenvalues for the general linear elliptic operator K follows as a corollary. In addition, it is shown that the eigenfunctions of K are complete in $L^p(D)$ for $p \leq \dfrac{2n}{n-2m}$ and closed in $L^1(D)$ for $2m > n$ while sharper estimates are given for the relative magnitudes of the real and imaginary parts of the eigenvalues.[4]

Although the discussion of this note is restricted to a single elliptic operator and to the Dirichlet boundary conditions, the method employed

may be extended to strictly elliptic systems of differential equations and general boundary-value problems. The detailed exposition of the application of this method in the more general case will be published elsewhere.[5]

The notation of the author's previous notes will be employed throughout except that functions considered will have complex values.

1. Let D be a bounded domain in E^n, $C^{2m}(D)$ the family of complex-valued functions $2m$-times continuously differentiable in D, $C_c^{2m}(D)$ the subfamily of $C^{2m}(D)$ of functions with compact supports in D, $L^2(D)$ the Hilbert space of complex-valued square-integrable functions on D with the usual inner product. (z^*, the complex conjugate of z.) If $f, g \in C_c^{2m}(D)$,

$$(f,g)_m = \int_D \left\{ \sum_{i_1 \ldots i_m} \frac{\partial^m f}{\partial x_{i_1} \ldots \partial x_{i_m}} \frac{\partial^m g^*}{\partial x_{i_1} \ldots \partial x_{i_m}} \right\} dx.$$

If $H_m(D)$ is the completion of $C_c^{2m}(D)$ with respect to $\| \quad \|_m$, the separable Hilbert space $H_m(D)$ may be identified in a unique fashion with a linear subset of $L^2(D)$ by a continuous extension of the identification mapping on $C_c^{2m}(D)$. The image of $H_m(D)$ will be described as the set of functions which together with their derivatives of order less than m vanish on the boundary of D in the variational sense.

Let K be a linear elliptic differential operator of order $2m$ on D, B a self-adjoint differential operator on D of order $2s$, $s < m$. B is said to be of a single sign provided that there exists $\rho > 0$ such that either $\int_D Bf \cdot f^* dx \geq \rho(f,f)_s$ for $f \in C_c^{2m}(D)$ or $\int_D Bf \cdot f^* dx \leq -\rho(f,f)_s$ for $f \in C_c^{2m}(D)$. The eigenfunctions of K with respect to B on D may be defined recursively as follows:

(1) If $\varphi \in C^{2m}(D) \cap H_m(D)$, ϕ is a first order eigenfunction of K with respect to B and with B-eigenvalue λ (λ, a complex number) if $K\phi - \lambda B\varphi = 0$.

(2) Let $\varphi \in C^{2m}(D) \cap H_m(D)$, $n > 1$. Then φ is an nth order eigenfunction of K with respect to B with B-eigenvalue λ provided that there exists a $(n-1)$ order eigenfunction ψ of K with respect to B with B-eigenvalue λ such that $K\varphi - \lambda B\varphi = B\psi$.

The first order eigenfunctions are the ordinary eigenfunctions of K with respect to B and the Dirichlet boundary conditions of D. If $\varphi \in C^{2m(n+1)}(D) \cap H_m(D)$ is a nth order eigenfunction of K with respect to B with B-eigenvalue λ and if K and B commute, then $(K - \lambda B)^n \varphi = 0$. This will always be the case when B is the identity operator on $C^{2m}(D)$ and the coefficients of K are sufficiently differentiable.

If $S \subset H_m(D)$, S is said to be complete in $H_m(D)$ if the family of finite linear combinations of elements of S with complex coefficients is dense in $H_m(D)$.

THEOREM 1. *Let K be a suitably differentiable linear elliptic differential operator of order $2m$ ($m \geq 1$) on the bounded domain D of E^n, B a suitably*

differentiable self-adjoint differential operator of a single sign on D of order $2s$, $s < m$. If $4m - 2s \leq n$, we suppose that the boundary of D is locally an $(n-1)$-manifold of class C^{2m}, $2m$-times continuously differentiably imbedded in E^n. Then the eigenfunctions of K with respect to B on D are complete in $H_m(D)$ and a fortiori in $L^2(D)$.

Proof: Since B is of a single sign on D, from the results of the author's previous notes[6] by considering $K + k_0 B$ for suitable k_0 instead of K, it may be assumed without loss of generality that the Dirichlet problem for K on D has no non-trivial null solutions. K may be written in the form $K = L + A$, where L is positive and self-adjoint, A of order less than $2m$, $\bar{A}$ (the formal adjoint of A) $= -A$, while $\int_D (-1)^m Bf \cdot f^* \, dx \geq \rho(f, f)_s$ for $f \in C_c^{2m}(D)$. For $f, g \in C_c^{2m}(D)$, set $(f, g) = (-1)^m \int_D Lf \cdot g^* \, dx$. The inner product $(,)$ may be extended to $H_m(D)$ and yields a Hilbert space $H(D)$ on that set whose norm $\| \ \|$ is equivalent to $\| \ \|_m$. There exist completely continuous linear transformations U, V of $H(D)$ uniquely defined by the conditions $(Uf, g) = \int_D Af \cdot g^* \, dx$, $(Vf, g) = \int_D Bf \cdot g^* \, dx$ for $f, g \in C_c^{2m}(D)$. Let $U_1 = (-1)^m U$, $V_1 = (-1)^m V$. $V_1^* = V_1$, $U_1^* = -U_1$, and V_1 is a positive, self-adjoint, completely continuous linear transformation of the Hilbert space $H(D)$. ($\|U_1 \psi\| \leq c \|\psi\|_{m-1}$, for a suitable $c > 0$, $\psi \in H(D)$.)

If $\varphi, \psi \in H(D)$, $\psi \in C^{2m}(D)$, then $\varphi \in C^{2m}(D)$ and $K\varphi - \lambda B\varphi = B\psi$ if and only if $(I + U_1)\varphi - \lambda V_1 \varphi = V_1 \psi$. Since the Dirichlet problem for K on D has no non-trivial null solutions, $(I + U_1)$ is non-singular. Let $T_1 = V_1(I + U_1)^{-1}$. By a simple inductive argument, φ is a nth order eigenfunction of K with respect to B on D with B-eigenvalue λ if and only if $\varphi \in H(D)$ and $\eta = (I + U_1)\varphi$ is a nth order characteristic vector of the linear transform T with characteristic value $1/\lambda$, i.e., $T\eta - \lambda^{-1}\eta = \theta$, where $\theta = 0$ if $n = 1$, θ is a $(n-1)$-st order characteristic vector of T with characteristic value $1/\lambda$ if $n > 1$. To show the completeness of the eigenfunctions of K with respect to B, it suffices to show the completeness of the characteristic vectors of T.

Let $\mu > 0$, $U_\mu = (I + \mu V_1)^{-1} U_1$, $V_\mu = (I + \mu V_1)^{-1} V_1$. Then it may be shown that η is a nth order characteristic vector of T with characteristic value ν if and only if $\varphi = (I + U_\mu)(I + U_1)^{-1}\eta$ is a nth order characteristic vector of $T_\mu = V_\mu(I + U_\mu)^{-1}$. It is, therefore, sufficient to prove the completeness of the eigenfunctions of any such T_μ.

LEMMA 1. *Given $\epsilon > 0$, for sufficiently large μ, $\|U_\mu\| < \epsilon$.*

Proof of Lemma 1: Let $\{\varphi_i\}$ be a sequentially ordered, complete orthonormal set of eigenfunctions of the positive, self-adjoint, completely continuous linear operator V_1 on $H(D)$. For each integer $r \geq 1$, let E_r be the linear subspace of $H(D)$ spanned by the first r of the $\{\varphi_i\}$, Q_r the projection on E_r, $(I - Q_r)$ the projection on $E_r^\perp$. Let $k_r = \{\sup \|(I - Q_r)U_1\psi\| \mid \psi \in E_r, \|\psi\| = 1\}$, $y_r = \{\sup \|U_1\psi\| \mid \psi \in E_r^\perp, \|\psi\| = 1\}$.

We assert that k_r, $y_r \to 0$ as $r \to +\infty$. It suffices to show that $y_r \to 0$ since for $\psi \in E_r$, $\psi_1 \in E_r^{\perp}$, $||\psi|| = 1$, $||\psi_1|| = 1$, we have $|(U_1\psi, \psi_1)| = |-(\psi, U_1\psi_1)| \leq y_r$ implying that $k_r \leq y_r$.

Suppose that y_r did not converge to zero as $r \to +\infty$. Then there exists $\alpha > 0$ and a sequence $\{\psi_r\}$, $\psi_r \in E_r^{\perp}$ for each r, for which $||U_1\psi_r|| \geq \alpha$, $||\psi_r|| = 1$ for all r. Since $\{\psi_r\}$ is an infinite sequence from $H_m(D)$ with uniformly bounded m-norm, by the Rellich selection theorem[7] there exists an infinite subsequence $\{\psi_{r_i}\}$ and $\psi_0 \in H_{m-1}(D)$ such that $\psi_{r_i} \to \psi_0$ in $(m-1)$-norm. For each fixed k, $(\psi_{r_i}, \varphi_k) \to 0$ as $i \to +\infty$. On the other hand

$$(\psi_{r_i}, \varphi_k) = \frac{1}{\tau_k}(\psi_{r_i}, V_1\varphi_k) = \frac{1}{\tau_k}\int_D \psi_{r_i} \cdot \bar{B}\varphi_k^* \, dx.$$

Since $s \leq m-1$, convergence of a sequence from $H_m(D)$ in $(m-1)$-norm implies its convergence in s-norm and consequently

$$\int_D \psi_0 \bar{B}\varphi_k^* \, dx = \lim_{i \to \infty} \int_D \psi_{r_i} \bar{B}\varphi_k^* \, dx = 0.$$

Since $\{\varphi_i\}$ is complete in $H_m(D)$, it is complete in $H_s(D)$. For $f, g \in C_c^{2m}(D)$, $\int_D f \cdot \bar{B}g^* \, dx$ defines an inner product which may be extended to $H_s(D)$ and yields a norm equivalent to the s-norm. It follows by a continuity argument that $||\psi_0||_s = 0$. Therefore $\{\psi_{r_i}\}$ converges to 0 in s-norm and, since the mapping of $H_{m-1}(D)$ into $H_s(D)$ is one to one, it converges to zero in $(m-1)$-norm. Thus $\alpha = 0$ which is a contradiction. Therefore k_r, $y_r \to 0$ as $r \to +\infty$.

Choose r_0 so large that k_{r_0}, $y_{r_0} < \epsilon/4$. As a transformation of E_{r_0} into itself $(I + \mu V_1)^{-1}$ has norm $1/\lambda + I_{r_0}\mu$ where τ_{r_0} is the least characteristic value of V_1 corresponding to the first r_0 characteristic vectors $\{\varphi_i\}$. Since all the eigen values of V_1 are positive, $||(I + \mu V_1)^{-1}|| \leq 1$ on $H(D)$. But,

$$(I + \mu V_1)^{-1} U_1\psi = (I + \mu V_1)^{-1} Q_{r_0} U_1 Q_{r_0}\psi + (I + \mu V_1)^{-1}(I - Q_{r_0}) U_1 Q_{r_0}\psi + (I + \mu V_1)^{-1} U_1 (I - Q_{r_0})\psi$$

Therefore,

$$||(I + \mu V_1)^{-1} U_1|| \leq ||U_1||/(1 + \tau_{r_0}\mu) + ||(I + \mu V_1)^{-1}||k_{r_0} + ||(I + \mu V_1)^{-1}||y_{r_0} \leq ||U_1||/(1 + \tau_{r_0}\mu) + k_{r_0} + y_{r_0} < \epsilon$$

for μ sufficiently large. Q. E. D.

A (not necessarily self-adjoint) linear transformation of the Hilbert space H is said to be of Hilbert-Schmidt type if there exists a complete orthonormal set $\{\psi_i\}$ such that $\sum_i ||T\psi_i||^2 < \infty$.[8]

LEMMA 2. *For every positive μ, $(T_\mu^*)^n$ is of Hilbert-Schmidt type, provided that the boundary of D satisfies the smoothness conditions of the hypothesis of Theorem 1. In case $4m - 2s \geq n + 1$, T_μ^* is itself of Hilbert-*

Schmidt type without any assumption on the smoothness of the boundary of D.

The proof of Lemma 2 depends upon an extension of the regularity properties established for the Green's function of K on D and its iterates $G_r(x, z)$.[9] If $4m - 2s \geq n + 1$, the lemma follows from the minimax principle for the eigenvalues of self-adjoint completely continuous linear operators and the properties of polyharmonic equations on the interior of an n-sphere. (If $R_\mu = (I + \mu V_1)^{-1} T_\mu^* (I + \mu V_1)$, $\theta \in C^{2m}(D)$, then $\varphi = R_\mu \theta$ if and only if $\varphi \in C^{2m}(D) \cap H(D)$, $K\varphi + \mu B\varphi = B\theta$.)

Choose μ so large that $\|U_\mu\| < 1/2 \sin(\pi/4n)$.

Let $\mathfrak{E}$ be the closed linear subspace of $H(D)$ spanned by the characteristic vectors of T. By the Riesz theory of completely continuous linear operators on a Banach space,[10] if ζ is a characteristic value of T_μ, there is a least integer $\nu(\zeta)$, the index of ζ, such that $(T_\mu^* - \zeta^* I)$ is a non-singular linear transformation of $(T_\mu^* - \zeta^* I)^{\nu(\zeta)}(H(D))$ onto itself. Let $H_0 = \bigcap_\zeta (T_\mu^* - \zeta^* I)^{\nu(\zeta)}(H(D))$, the intersection taken over the characteristic values of T. Then H_0 is a closed subspace of $H(D)$ being the orthogonal complement of $\mathfrak{E}$ in $H(D)$, H_0 is invariant under T^*, and T^* has no characteristic values on H_0. To complete the proof of Theorem 1, we shall assume that $H_0 \neq \{0\}$ and deduce a contradiction.

Let P_0 be the projection of $H(D)$ on H_0, $V_0 = P_0 V_\mu P_0 | H_0$, $U_0 = P_0 U_\mu P_0 | H_0$. By choice of μ, $\|U_\mu\| < 1$ and therefore $T_0 = (I - U_0)^{-1} V_0$ is well defined as a linear operator on H_0 and coincides with T_μ^* on H_0. By assumption T_0 does not have any eigenvalues on H_0. For each complex number ζ which is not an eigenvalue of V_0,

$$(T_0 - \zeta I)^{-1} = (V_0 - \zeta I)^{-1}(I - U_0) - \zeta (V_0 - \zeta I)^{-1} U_0 (T_0 - \zeta I)^{-1}.$$

For all ζ such that $\|(V_0 - \zeta I)^{-1}\| \, \|U_0\| \leq 1/2$,

$$(\gamma) \quad \|(T_0 - \zeta I)^{-1}\eta\| \leq \|2(V_0 - \zeta I)^{-1}(I - U_0)\eta\|$$

for each $\eta \in H_0$. Since V_0 is a positive self-adjoint, completely continuous linear operator on H_0, $\|(V_0 - \zeta I)^{-1}\| \leq |Im(\zeta)^{-1}|$. The subset of all ζ in the complex plane for which (γ) holds contains the set whose complement is the union with the origin of the interior of an angular sector S at the origin whose angle is less than $\pi/2n$. But,

$$(\zeta \neq 0), (T_0 - \zeta I)^{-1} = \frac{1}{\zeta} \left\{ \sum_{j=0}^{n-1} \zeta^{-j} T_0^j \right\} \zeta^n (T_0^n - \zeta^n I)^{-1}.$$

By Lemma 2, $(T_0)^n$ is a linear operator of Hilbert-Schmidt type and, therefore, by an extension of a theorem due to Carleman, on integral operators of Hilbert-Schmidt type, since T_0^n has no eigenvalues, there exists $a > 0$ such that for sufficiently small ξ, $\|(T^n - \xi I)^{-1}\| \leq e^{a\xi^{-2}}$.[11] Therefore, $\|(T_0 - \zeta I)^{-1}\| \leq e^{a\zeta^{-2n}}$ for small ζ. Let $\eta_i = (I - U_0)\varphi_i$.

On the boundary of S except at 0,

$$|(T_0 - \zeta I)^{-1}\eta_i, \varphi_j)| \leq \frac{||I - U_0||}{|\tau_i - \zeta|},$$

τ_i real. Since it is analytic in ζ on the whole complex sphere except at 0, by a classical theorem of Phragmén-Lindelof type, $(T - \zeta I)^{-1}\eta_i, \varphi_j)$ is uniformly bounded in the angular sector,[12] and therefore is a constant in ζ. For $|\zeta| \to \infty$, $|((T - \zeta I)^{-1}\eta_i, \varphi_j)| \to 0$ and therefore is equal to 0. Since this is true for all j, $\eta_i \neq 0$, $(T - \zeta I)^{-1}\eta_i = 0$ which is a contradiction. Q. E. D.

2. The completeness theorems of this and the previous notes were established for $H_m(D)$ and include completeness in $L^2(D)$. In every case, however, a properly stronger conclusion than L^2-completeness may be derived.

A subset S of $L^p(D)$ is complete if its finite linear combinations with complex coefficients are dense. $S \subset L^1(D)$ is closed in $L^1(D)$ if $\int_D f \cdot \varphi^* dx = 0$ for all $\varphi \in S$ implies $f = 0$ a.e. in D.

THEOREM 2. *Let K be a suitably differentiable linear elliptic differential operator of order $2m$ on the bounded domain D of E^n, B a suitably differentiable, differential operator on D of a single sign, of order less than $2m$. Then:*

(a) If $2m > n$, the eigenfunctions of K with respect to B on D are closed in $L^1(D)$ and, therefore, are complete in $L^p(D)$ for every p.[13]

(b) Let $2m \leq n$. Suppose the domain D is smoothly bounded as in the hypothesis of Theorem 1. Then the eigenfunctions of K with respect to B are complete in $L^p(D)$ for $p \leq 2n/n - 2m$ (∞ if $n = 2m$).

Proof: Theorem 2 follows from Theorem 1 and the following partially strengthened form of two theorems due to S. Soboleff:[14]

(α). If D is a bounded domain, $x_0 \in D$, the linear functional $\sigma_{x_0}(f) = f(x_0)$ defined for $f \in C_c^{2m}(D)$ is bounded in the m-norm if $2m > n$. Furthermore, $||\sigma_{x_0}|| \leq M$ for a suitable $M > 0$ and all $x_0 \in D$.

(β). If D is a smoothly bounded domain, $p \leq 2n/(n - 2m)$, the mapping of $C_c^{2m}(D)$ into $L^p(D)$, induced by the natural identification, is a bounded linear transformation in the m-norm.

Completeness in $L^p(D)$ follows in both cases. If $2m > n$, (α) implies that every $f \in C_c^{2m}(D)$ can be approximated uniformly on D by finite linear combinations of the eigenfunctions of K.

Therefore $\int_D f \cdot g^* dx = 0$ for all $g \in C_c^{2m}(D)$ implying that $f = 0$ almost everywhere in D.

THEOREM 3. *Let K be a suitably differentiable linear elliptic differential operator of order $2m$ on the bounded domain D of E^n, B a self-adjoint differential operator of order $2s$ which is of a single sign and suitably differentiable on D ($s < m$). Let $K = L + A$, where L is self-adjoint and A is of order $q < 2m$.*

Then if $\lambda_i = \xi_i + i\eta_i$ *is the sequence of distinct eigenvalues of* K *with respect to* B, $(-1)^m\xi_i \to +\infty$ *if* $\int_D Bf \cdot f^* \, dx$ *is assumed positive for all* $f \in C_c^{2m}(D)$. *There exists* $\xi_0 > 0$, $c > 0$, *such that for all* i

$$\begin{aligned} |\eta_i| &\leq c\{(-1)^m\xi_i + \xi_0\}^{\frac{q-2s}{2m-2s}} && \text{for } q > 2s \\ |\eta_i| &\leq c && \text{for } q \leq 2s \end{aligned}$$

Proof: By integration by parts we obtain $||f_j|| \leq ||f||_{j+1}^{1/2} \cdot ||f||_{j-1}^{1/2}$ for $f \in C_c^{2m}(D)$ and therefore for $f \in H_m(D)$ provided that $j + 1 \leq m$. By induction on $m - s$, it follows that for $s < j < m$,

$$||f||_j \leq ||f||_s^{\binom{m-j}{m-s}} \, ||f||_m^{\binom{j-s}{m-j}} \text{ for } f \in H_m(D).$$

The remainder of the proof follows that of Theorem 1 of the author's second note.

[1] Browder, F. E., (I) "The Dirichlet Problem for Linear Elliptic Equations of Arbitrary Even Order with Variable Coefficients," PROC. NATL. ACAD. SCI., **38,** 230–235 (1952); (II) "The Dirichlet and Vibration Problems for Linear Elliptic Differential Equations of Arbitrary Order," *Ibid.*, **38,** 741–747 (1952); (III) "Assumption of Boundary Values and the Green's Function in the Dirichlet Problem for the General Linear Elliptic Equation," *Ibid.*, 179–184 (1953); (IV) "Linear Parabolic Differential Equations of Arbitrary Order; General Boundary-Value Problems for Elliptic Equations," *Ibid.*, 185–190 (1953).

[2] Cf. ref. 1 (II)

[3] For ordinary differential operators, cf. Birkhoff, G. D., Collected Works. The author has been informed by P. Lax that the latter and J. Berkowitz have obtained results similar to ours for second-order operators and that their methods can be extended to higher order operators for the case $B = I$.

[4] This is an improvement of Theorem 2 of (II).

[5] Browder, F. E., "Linear Elliptic Systems of Differential Equations," to appear elsewhere.

[6] Cf. ref. 1 (I), (II).

[7] Courant-Hilbert, *Methoden der Mathematische Physik*, Vol. 2, pp. 488–490, Berlin, 1937.

[8] Cf. Smithies, F., *Duke Math. J.*, **8,** 107–130 (1941).

[9] Cf. ref. 1 (III), (IV).

[10] Riesz, F., *Acta. Math.*, **41,** 71–98 (1917).

[11] Carleman, T., *Math. Zeit.*, **9,** 196–217 (1921).

[12] Titchmarsh, E. C., *The Theory of Functions*, Oxford, 1932.

[13] This includes the theorem for ordinary differential operators of Levinson, N., *Duke Math. J.*, **19,** 23–26 (1952).

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ON DIFFERENTIAL GEOMETRY IN THE LARGE*

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Differential geometry in the large is the study of certain analytical systems arising from problems of a geometrical nature together with the manifolds which bear those systems, these manifolds being in fact the totality of parameter systems in which the analytical structures are to be examined; in other words, it is a natural extension of classical domains of analysis viewed under a geometric aspect. In that sense the most natural problem in differential geometry in the large is the investigation of the *effect of the particular nature of an analytical system on the nature of its manifold*. Now by virtue of the importance of its applications and natural origins the most significant such analytical object is Riemannian "geometry."

In the light of the preceding remarks I should like to present here in the form of a theorem (Theorem 1 below) a result which is definitive within the cadre of presently available methods of analysis and knowledge about specific examples of Riemannian manifolds. Some preliminary explanation is essential for comprehension and evaluation of the theorem.

By far the most fruitful approach to the study of a Riemannian manifold in the large from the stated point of view proceeds via the examination of the geodesics, since they determine the geometrical form of the manifold and are, in turn, determined by the metric and, in particular, by the curvature, its principal invariant. Thus it was quite simple for Hadamard and Cartan to show that all manifolds of non-positive curvature have a topologically Euclidean space as universal covering by showing that the geodesics issuing from a fixed point fan out like straight lines. One sees then that the essential difficulties, as Poincaré once remarked, arise in manifolds of positive curvature, since then the geodesics curve in on one another and intersect in the most complex fashion.

To gain an idea of what is known about manifolds of positive curvature and to see what metric restrictions might be necessary to draw definitive conclusions about their geometry it is enough to refer to all the known examples, these being a subclass of that large and remarkable class of manifolds discovered by Elie Cartan and called by him *symmetric spaces*.[1] Those in question are, in essence, the closed, simple Lie groups and their co-set spaces. Their differential-geometric and geodesic structures (in particular, their topology) are completely accessible, if not explicitly written out. The geodesics are the orbits of one-parameter subgroups, and the characteristic property which will be critical in what follows is that *the curvature of any two-section is preserved by parallel displacement*. From

the analytic viewpoint the underlying manifold of a symmetric space is simply the totality of solutions of an *integrable* (in the sense of Jacobi or Lie) set of ordinary differential equations—the equations of the geodesics—or a *completely integrable* set of partial differential equations—the equations of the infinitesimal transformations. As far as differential geometry in the large is concerned the salient fact is that *the rigid local integrable metric structure completely determines the topological structure;* one can reproduce the space, as it were, by integration and analytic continuation.

Now one might suspect that the very special geometrical (i.e., topological) structure of the examples is due exclusively to the special integrability of their metric structures and that, therefore, their multitude and complexity indicate that such integrability is essential to produce so decisive an effect on the topology; or, to put it another way, that a slight perturbation or disturbance which would destroy the integrability would upset the behavior of the geodesics and thus alter the topological structure.

But I have discovered that that is not so, and the discovery exposes the real nature of the influence of the differential geometry of a manifold on its topology, not to mention the true role played by the symmetric spaces in differential geometry.

The geodesic or topological structure of a symmetric space is in fact a result not of special integrability but of two very natural differential-geometric conditions of a far less restrictive nature. To explain one of these I must recall the concept of holonomy group[2] of a Riemannian manifold M^n; namely, the subgroup of the orthogonal group $0(n)$ consisting of all the linear transformations of the tangent space T_p at $P \in M^n$ onto T_p at P, generated by parallel displacement of a frame in T_p along all paths joining P and P_1. Then my result says roughly that given any compact simply connected symmetric space $\mathcal{E}^n$ with holonomy group $\mathfrak{K}$ then any Riemannian M^n, with holonomy group H no larger than $\mathfrak{K}$, whose curvature differs from that of $\mathcal{E}^n$ at most by numerical limits depending only on $\mathcal{E}^n$ has universal covering $\tilde{M}^n$ homeomorphic with $\mathcal{E}^n$; i.e., within the class of M^n, with at most a certain mobility under parallel displacement, the curvature acts as a gauge which picks out $\mathcal{E}^n$ (topologically) when it falls within certain limits. Thus the hypotheses allow for the wildest sort of local variation of analytic structure. The need for simple connectedness is shown clearly by the example of the Clifford-Klein space-forms which have identical metrics but differing fundamental groups. In analytical terms the theorem is really a far-reaching analog of the theorem of Poincaré[3] to the effect that a periodic solution of a set of differential equations involving a parameter μ admits analytic continuation in μ if no characteristic exponent is zero. It was the general idea of analytic continuation, of course, that led to the discovery of my theorem.

In precise formulation one has

THEOREM 1. *Let $\mathcal{E}^n$ $(n \geq 2)$ be an n-dimensioncl, simply connected, symmetric Riemannian manifold of positive (normalized) curvature, with holonomy group $\mathfrak{H}$. Then there exists a constant $0 < c(\mathcal{E}^n) < 1$ of the following nature: if M^n, a complete n-dimensional Riemannian manifold of class C_3 and holonomy group H, is such that (1) $H \subseteq \mathfrak{H}$, (2) there exists a transformation $h_p \in \mathfrak{H}$ of the tangent space T_p at $P \in M^n$ onto the tangent space $\mathfrak{Z}$ at a fixed point of $\mathcal{E}^n$ under which*

$$c(\mathcal{E}^n)\mathcal{K}(h_p\gamma) < K(\gamma, P) < \mathcal{K}(h_p\gamma)$$

(where γ is a 2-element in T_p, $h_p\gamma$ its image in $\mathfrak{Z}$, $K(\gamma, P)$ the curvature of M^n at P in the direction γ, $\mathcal{K}(h_p\gamma)$ the corresponding curvature of $\mathcal{E}^n$); *then the universal covering $\bar{M}^n$ of M^n is homeomorphic to $\mathcal{E}^n$.*

The main theorem of my paper *A contribution to differential geometry in the large*[4] is the special case of Theorem 1 when $\mathcal{E}^n = S^n$, the n-dimensional sphere, and $c(S^n) \sim 0.75$. I have found that when $\mathcal{E}^{2n} = P^n$, complex projective space of n-dimensions, $c(P^n) \sim 0.95$.

Indeed, the basic ideas of proof are already contained in the aforesaid paper, an essential refinement in the analytic technique thereof involving Hamilton's principle of varying action and more detailed geometric reasoning being necessary for the general theorem. The main device now as before is the duplication of the geodesic structure of $\mathcal{E}^n$ in M^n by means of broken geodesics and the formation of the obvious correspondence.

It is my intention to publish details and proofs in a subsequent series of publications.

* I have been in possession of these results since 1951, having presented them at various colloquia.

[1] *Oeuvres Complètes*, Élie Cartan, Paris, 1952, part 1, vol. 2, p. 1247 and references there.

[2] *Ibid.*, p. 841.

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A CLASSICAL MODEL OF A ROTON

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1. Introduction.—In his theory of liquid Helium II, Landau[1] assumes that, in addition to the phonons associated with compressional waves, the liquid possesses another degree of freedom manifested by vortex motion. The vortex motion he assumes to be quantized, and he applies to it the term rotons. Since the angular momentum of a roton cannot be arbitrarily small, but must be of the order of $\hbar$, he concludes that between the ground state of the phonons and that of the rotons there is an energy-gap Δ. From dimensional considerations it follows[2] that

$$\Delta \sim \rho^{2/3}\hbar^2 m_{\mathrm{He}}^{-5/3}, \tag{1}$$

which is of the order of $1^\circ k$, where k is Boltzmann's constant. He is thus led to the assumption that for small values of the momentum p of the roton, the energy of the roton E is given by

$$E = \Delta + p^2/2\mu, \tag{2}$$

where μ is an effective mass of the roton. The values of Δ and μ he determined empirically using Keesom and Keesom's[3] data on the specific heat of Helium II, obtaining

$$\Delta/k = 8 - 9^\circ, \qquad \mu = 7 - 8m_{\mathrm{He}}. \tag{3}$$

Subsequent measurements of the velocity of second sound by Peshkov[4] having shown that μ varies with temperature, Landau[5] modified expression (2) to the form

$$E = \Delta + (p - p_0)^2/2\mu, \tag{4}$$

with

$$\Delta/k = 9.6^\circ, \quad p_0 = 2.06 \times 10^{-19}, \quad \mu = 0.77m_{\mathrm{He}} = 5.2 \times 10^{-24}\ \mathrm{g}. \tag{5}$$

Khalatnikov[6] has reevaluated these constants, using more recent measurements of specific heat and velocity of second sound in Helium II, obtaining

$$\Delta = (8.9 \pm 0.2)^\circ, p_0 = (2.1 \pm 0.05) \times 10^{-19}, \mu = (1.72 \pm 0.68) \times 10^{-24}\ \mathrm{g}. \tag{6}$$

It would seem from Landau's expressions for the thermodynamic functions that p in (4) denotes $|p|$.

2. Hicks Spherical Vortex.—In attempting to construct a model of a roton one meets with the difficulty that so far it has not been demonstrated that

vortex motion can have a wave-character analogous to sound propagation in the case of phonons. The picture of isolated vortex lines, which are conserved by the fluid particles along their path, is also not acceptable since it involves singularities in the velocities and in the pressure on the vortex lines. A vortex ring with finite velocities everywhere provides a classical model of an isolated system endowed with kinetic energy of vortical origin. However, in an ordinary vortex ring there is no net angular momentum, which one would like to attribute to a roton. If the path of a particle in a vortex ring were not limited to one meridional plane, but were a spiral coiled around the surface of a torus, the system would possess an angular momentum. This is a characteristic feature of the spherical vortex described by Hicks.[7]

It will be helpful to consider first the motion induced in an ideal incompressible fluid by a rigid sphere advancing with constant velocity U. The motion is irrotational and is derived from a stream-function ψ_0.

$$\psi_0 = \pi U \sin^2\theta[(a^3/r) - r^2], \tag{7}$$

$$u_r = (1/2\pi r^2 \sin\theta)\ (\partial\psi/\partial\theta);\quad u_\theta = -(1/2\pi r \sin\theta)\ (\partial\psi/\partial r),\ u_\varphi = 0. \tag{8}$$

Here we have used a system of coordinates with origin at the center of the sphere. At great distances from the sphere the liquid moves past the sphere with constant velocity U from the direction $\theta = 0$ toward $\theta = \pi$. To obtain the motion when the liquid is at rest at infinity, the r^2 term in (7) is to be dropped. The fluid motion produces a variable pressure on the surface of the sphere which is positive at the poles and reaches a maximum negative value on the equator.

If the rigid sphere were now replaced by the liquid, the latter would be set into internal motion due to the variable pressure produced by the liquid outside the sphere. One may inquire whether it is possible to find a motion of the liquid inside the sphere which shall be continuous, and which at the surface of the sphere shall match the velocities and pressures in the fluid outside. That this is possible was demonstrated by Hill.[8] In Hill's spherical vortex the motion inside the sphere is in meridional planes, like in a vortex ring. The system has no angular momentum.

In the Hicks[7] spherical vortex the fluid inside the sphere has also a longitudinal component of velocity. The meridional components of velocity are still derivable from a stream-function ψ by the relations given in (8), but ψ now has the form

$$\psi_i = A \sin^2\theta\ [(\sin\lambda x/\lambda x) - \cos\lambda x - x^2 J'], \tag{9}$$

$$x = r/a, \qquad J' = [(\sin\lambda/\lambda) - \cos\lambda], \tag{10}$$

$$A = (3M/4\rho a^2\lambda)/s(\lambda); \qquad s(\lambda) = \left[\left(\frac{3}{\lambda^2} - \frac{1}{5}\right) J' - \frac{\sin\lambda}{\lambda}\right], \tag{11}$$

and there is a longitudinal component of velocity $u_\varphi = \lambda\psi/2\pi ar \sin\theta$. Here λ is a dimensionless parameter, and M is the integral of the angular momentum over the sphere

$$M = 2\pi \int_0^a r^3\, dr \int_0^\pi \sin^2\theta u_\varphi\, d\theta. \tag{12}$$

This is also the total angular momentum of the system, since outside the sphere the motion is represented by (7) and (8), with u_φ vanishing. On the surface of the sphere u_φ vanishes, and the components u_r and u_θ match the values of the potential motion outside. The pressure is therefore also continuous. The velocity of advance U of the sphere relative to the fluid at infinity is given by

$$U = U_0 q(\lambda); \qquad U_0 = (3M/4\pi\rho a^4); \qquad q(\lambda) = [(J'/\lambda) - (1/3)\sin\lambda]/s(\lambda). \tag{13}$$

Computing the kinetic energy of the fluid, both outside and inside the sphere, in a coordinate system in which the fluid is at rest at infinity, we get

$$E = E_0 L(\lambda), \qquad E_0 = (9M^2/16\pi\rho a^5), \tag{14}$$

$$L(\lambda) = \left[\left(\frac{2}{5} - \frac{2}{\lambda^2}\right) J'^2 - \frac{2}{3\lambda}\sin\lambda J' + \frac{4}{9}\sin^2\lambda\right]/s^2(\lambda). \tag{15}$$

With a given value of the parameter λ one can compute $q(\lambda)$ and $L(\lambda)$ from (13) and (15), and thus obtain a relation between ϵ and v, where

$$\epsilon = E/E_0, \qquad v = U/U_0. \tag{16}$$

In this way one obtains the dependence of the energy of the system on the momentum *for a given value of the angular momentum* M. For small values of λ, U is large, and since M is fixed, this means that the energy is predominantly non-rotational. ϵ is then proportional to v^2. As λ is increased, the energy goes through a minimum, as shown in figure 1.

We notice that for $v = 0$, ϵ is not zero but is equal to 10. This corresponds to the value $\lambda = 5.76$, when the numerator of $q(\lambda)$ vanishes. For this value of the parameter we have a spherical vortex which is stationary in the fluid. On the surface of the sphere all three components of velocity vanish, and with them the dynamic pressure. There is no motion outside the sphere. At points outside the sphere there is therefore no way of telling of the existence of the vortex.

For λ up to 5.76 there is one ring, of radius less than a, lying in the equatorial plane which threads through all the trajectories. Looking at the vortex along the direction of advance **U**, the fluid moves forward inside this ring while it regredes outside. The longitudinal motion is such that the momentum vector **M** is directed along **U**.

As λ is increased beyond 5.76, **U** becomes negative relative to the direction of the momentum vector **M**. What happens is that from the surface of the sphere down to a certain depth there appears a shell with a second equatorial ring which is threaded in a direction opposite to that of the equatorial ring in the nucleus. The longitudinal motion in the shell is opposed to that in the nucleus, but the net angular momentum for the whole sphere

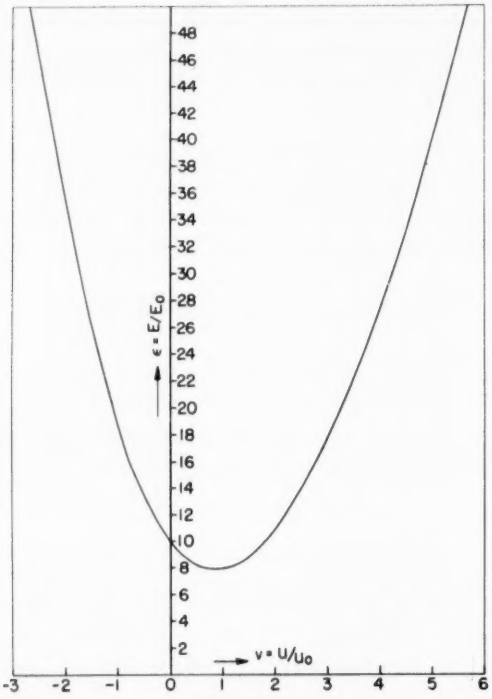

FIGURE 1

Energy E of a Hicks spherical vortex as a function of the velocity U for a given value of the angular momentum M in the first mode. $E_0 = (9M^2/16\pi\rho a^5)$, $U_0 = (3M/4\pi\rho a^4)$, a = radius of sphere.

is still of the sign of the angular momentum in the nucleus. The motion inside the equatorial ring of the nucleus regredes relative to the direction of advance.

The energy curve shown in figure 1 we shall refer to as the first mode. As λ is further increased, one finds higher modes, the first three being shown in figure 2. They all cross the ϵ-axis at the value 10. Each curve has a

minimum at some positive value $v_{\min.}$, and $v_{\min.}$ decreases with increasing order of the mode. For positive v the first mode lies below all the higher ones, while for negative v it is above all the higher modes.

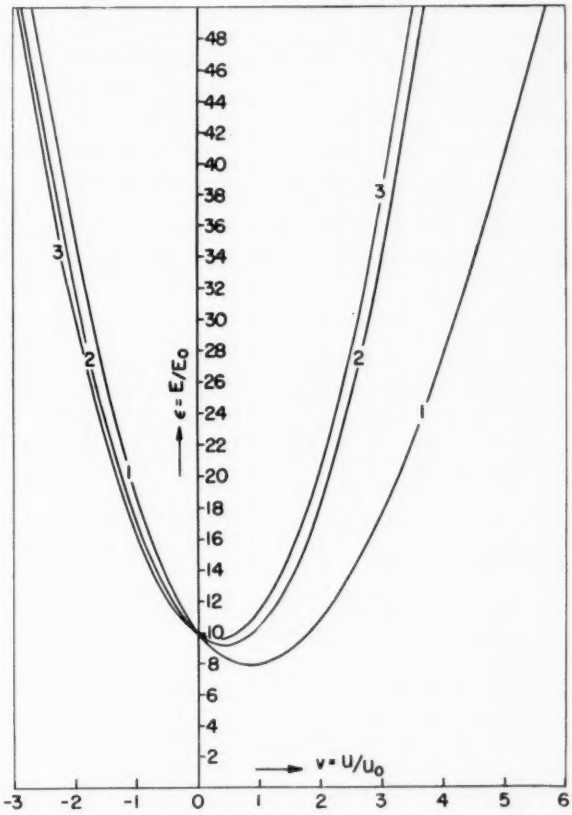

FIGURE 2

Energy E of a Hicks spherical vortex as a function of the velocity U for a given value of the angular momentum M in the first three modes. $E_0 = (9M^2/16\pi\rho a^3)$, $U_0 = (3M/4\pi\rho a^4)$, a = radius of sphere.

Near the minimum we can write

$$L = \alpha + \beta(q - q_0)^2, \tag{17}$$

$$\alpha_1 = 7.905, \qquad \beta_1 = 2.778, \qquad q_0^{(1)} = 0.842. \tag{18}$$

For large values of the order of the mode n

$$\alpha \to 10 - (5.7/n^2), \quad \beta \to 4(1-0.46/n^2), \quad q_0 \to 1.2/n. \quad (19)$$

The above discussion applies to positive values of λ. For negative values of λ we have another Hicks spherical vortex which differs from the one corresponding to positive values of λ only through the reversal of the sense of longitudinal motion. The modes are therefore doubly degenerate.

3. Applications to Rotons.—In the following we shall investigate the extent to which the Hicks spherical vortex described above can be adopted as a model for rotons. Pending an investigation of the stability of the various modes of the spherical vortex, we shall consider only the first mode for which the dependence of energy on velocity of advance of the vortex is shown in figure 1. For low temperatures the portion of the curve near the minimum is decisive, so that we can represent the energy E of a roton by

$$E = (9M^2/16\pi\rho a^5)[\alpha + \beta(q - q_0)^2], \quad (20)$$

with the values of α, β, and q_0 given in (18). We wish to determine the energy as a function of the momentum of advance $\mathbf{p}$ of the vortex. The effective mass of the vortex is represented by the mass m of the fluid inside the sphere and by the induced mass in the fluid outside the sphere. The latter is $(1/2)m$. Hence

$$\mathbf{p} = (3/2)m\mathbf{U} = (3\mathbf{M}/2a)q. \quad (21)$$

Substituting into (20) we get

$$E = \Delta + (p - p_0)^2/2\mu. \quad (22)$$

$$\Delta = (9M^2\alpha/16\pi\rho a^5), \quad \mu = (3m/2\beta), \quad p_0 = (3M/2a)q_0. \quad (23)$$

Equation (22) is of the same form as the one adopted by Landau. However, where Landau has to determine the three constants Δ, μ, and p_0 from the observations, we have only two independent constants at our disposal: the angular momentum M and the radius of the sphere a.

Adopting equation (22) for the energy of a roton, we can determine the thermodynamic functions from the free energy F

$$F = \pm(kT/\rho)(8\pi/h^3)\int_{-\infty}^{\infty} \ln\left\{1 \mp \exp\left[-E(p)/kT\right]\right\} p^2\, dp. \quad (24)$$

Here the upper signs refer to Bose-Einstein, and the lower signs to Fermi-Dirac statistics. A factor of 2 was introduced because of the double-degeneracy. Because of the large value of Δ/kT, the exponential term in the integral is very small, so that the type of statistics does not matter. For positive λ the integration from 0 to ∞ applies to vortices whose angular momentum $\mathbf{M}$ points in the direction of the velocity of advance $\mathbf{U}$, while the integration from $-\infty$ to 0 applies to vortices in which $\mathbf{M}$ is anti-parallel

to **U**. The opposite is true for negative values of λ. Substituting (22) into (24), we get

$$F = -(4kT/\rho h^3)(2\pi k\mu T)^{3/2}e^{-x}(\omega x + 1). \quad (25)$$

$$\rho_n/\rho = -(8\pi/3h^3\rho)\int_{-\infty}^{\infty} p^4\,dp\,\frac{\partial}{\partial E}[\exp(E/kT) \mp 1]^{-1} = (4\mu/\rho h^3)(2\pi\mu kT)^{3/2}e^{-x}[1 + 2\omega x + \omega^2x^2/3]. \quad (26)$$

$$S_r = -\partial F/\partial T = (4k/\rho h^3)(2\pi k\mu T)^{3/2}e^{-x}[\omega x(1.5 + x) + 2.5 + x]. \quad (27)$$

$$c_r = T\partial S/\partial T = (4k/\rho h^3)(2\pi k\mu T)^{3/2}e^{-x}[\omega x(0.75 + x + x^2) + (3.75 + 3x + x^2)], \quad (28)$$

where S_r denotes the entropy of rotons, c_r the specific heat of rotons, ρ_n the density of the "normal fluid," and

$$x = (\Delta/kT); \qquad \omega = 2q_0^2\beta/\alpha = 0.498. \quad (29)$$

The specific heat and entropy of Helium II have recently been measured by Kramers, Wasscher, and Gorter,[9] and are given in table 1. Values of

TABLE 1.

THERMODYNAMIC FUNCTIONS OF HELIUM II

$S_r = S - 0.0078T^3$, $c_r = c - 0.0235T^3$. In the computed values the following constants were used: $M = 1.5h$, $a = 3.19 \times 10^{-8}$ cm., $m = 2.94m_{He}$, $\Delta = 8.93k°$.

T, °K.	S, JOULE/G. DEG., OBS.	S_r, JOULE/G. DEG., OBS.	S_r, JOULE/G. DEG. COMP.	c, JOULE/G. DEG., OBS.	c_r, JOULE/G. DEG., OBS.	c_r, JOULE/G. DEG., COMP.
0.60	0.00169	0.	0.	0.0051	0.0000	0.0004
0.70	0.00276	0.	0.00021	0.0098	0.0017	0.0026
0.80	0.00475	0.00075	0.00103	0.0222	0.0102	0.0109
0.90	0.00885	0.00315	0.00335	0.0510	0.0339	0.0326
1.00	0.0168	0.0090	0.0089	0.1042	0.0807	0.0780
1.10	0.0304	0.0201	0.0198	0.191	0.160	0.158
1.20	0.0523	0.0386	0.0385	0.322	0.281	0.284
1.30	0.0853	0.0680	0.0679	0.516	0.464	0.463
1.40	0.132	0.111	0.111	0.780	0.715	0.703
1.50	0.197	0.171	0.169	1.127	1.048	1.008
1.60	0.284	0.252	0.246	1.572	1.476	1.379
1.70	0.395	0.357	0.342	2.11	1.995	1.817
1.80	0.535	0.490	0.460	2.80	2.66	2.32
1.90	0.709	0.655	0.600	3.63	3.47	2.89
2.00	0.929	0.867	0.764	4.95	4.76	3.51
2.05	1.061	0.994	0.855	5.82	5.62	3.85
2.10	1.215	1.143	0.952	6.92	6.70	4.20
2.15	1.40	1.32	1.05	8.61	8.38	4.56
2.18	1.53	1.45	1.12	11.6	11.4	4.78
2.186	1.57	1.49	1.13	14.3	14.1	4.83

ρ_n/ρ can be taken from the direct measurements of Andronikashvilli,[10] or can be derived from the measured values of the velocity of second sound. The values of the parameters M and a which we are seeking have therefore to stand the test of fitting three sets of essentially independent types of experimental data. We shall determine them by fitting the entropy measurements. On plotting $\ln S_r$ against $1/T$, one finds that, except at the ends of the temperature range, the relationship is linear, with a slope corresponding to

$$\Delta/k = 8.93^\circ. \tag{30}$$

We now assume the angular momentum to be quantized

$$M = l\hbar, \qquad \Delta = 9M^2\alpha/16\pi\rho a^5 = 9l(l+1)\hbar^2\alpha/16\pi\rho a^5. \tag{31}$$

With Δ known, we can express a in terms of $l(l+1)$, and similarly μ $(= 2\pi\rho a^3/\beta)$. Substituting the latter in (27), we determine l by fitting with the observed value of S_r at 1.5°. The agreement is obtained at $l = 1.5$. From (31) it now follows that $a = 3.19 \times 10^{-8}$ cm., leading to the values

$$m = 2.94m_{\mathrm{He}}, \quad \mu = 1.59m_{\mathrm{He}}, \quad p_0 = 5.94 \times 10^7\hbar = 6.26 \times 10^{-20}, \tag{32}$$

$$S_r = 1.16T^{3/2}e^{-x}[0.498x(1.5+x) + 2.5 + x]\text{joule/g. deg.}; \qquad x = (8.93/T), \tag{33}$$

$$c_r = 1.16T^{3/2}e^{-x}[0.498x(0.75 + x + x^2) + 3.75 + 3x + x^2]\text{joule/g. deg.}, \tag{34}$$

$$\rho_n/\rho = 0.8967T^{3/2}e^{-x}(1 + 0.995x + 0.0826x^2). \tag{35}$$

It will be seen from table 1 that, except near the lambda points, the agreement between computed and observed values of S_r is satisfactory. The observed c_r agree with the computed values below 1.80°, but above that temperature the two deviate markedly. The computed values of ρ_n/ρ are too low by a factor of about 3. In comparing our computed values with those obtained by the use of Khalatnikov's parameters given in equation (6), we find that the latter are in better agreement for ρ_n/ρ, and are about the same for c_r and S_r. Our values are of course only preliminary, pending an investigation of the stability of the various modes, and of the role of the higher modes, whose contribution we have neglected.

4. *Discussion.*—It is not proposed that the classical spherical vortices discussed in this paper resemble closely the rotons in Helium II, which presumably owe their origin to purely quantum effects. It is suggested, however, that certain features exhibited by these vortices will survive in the roton theory of the future: (1) the angular momentum of a vortex is colinear with its momentum; (2) a vortex can have a finite energy with

zero momentum, due to its angular momentum; (3) a vortex has a minimum energy at some non-zero value of momentum.

* Part of this work was done at the Institute for Advanced Study in the Fall of 1952.

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